ENDOCRINOLOGY

VOLUME 62 APRIL,, 1958 NUMBER 4

Copyright © 1958 by the Endocrine Society

EFFECT OF DESOXYCORTICOSTERONE ACETATE ON METAMORPHOSIS INDUCED BY THYROXINE IN ANURAN TADPOLES

HIDESHI KOBAYASHI

Zoological Institute, Faculty of Science, Tokyo University, Tokyo, Japan

ABSTRACT

Effects of desoxycorticosterone acetate (DCA) on thyroxine-induced metamorphosis were studied. When tadpoles at hind limb-bud stage are treated with DCA alone, their metamorphosis is completely inhibited. DCA seems to inhibit development of functional activity of the thyroid gland in the tadpoles at this stage. But when thyroxine and DCA are given simultaneously, the steroid accelerates the water loss as well as the following degenerative changes of thyroxine-induced metamorphosis: decrease in the length of trunk and tail, resorption of tail fin and opercular perforation. On the other hand, it antagonizes slightly but significantly the effect of thyroxine on anabolic changes such as the growth of forelegs and hind limbs. It is suggested that the ratio of circulating amounts of thyroxine and mineralocorticoids is important in regulating the metamorphosis of anuran tadpoles.

IN A series of comparative studies on the effect of corticosteroids on the thyroid gland, we have found that desoxycorticosterone acetate (DCA) accelerates metamorphosis when it is administered to toad tadpoles with hind limbs of about 3 mm. in length, but it inhibits when given to tadpoles at hind limb-bud stage (1 and 2). Gasche (3) has made similar observations in *Xenopus*. This contradictory effect of DCA on metamorphosis is presumed to be associated with different levels of thyroid activity prevailing in the tadpoles at the two developmental stages. In favor of this supposition is the general agreement that the thyroid gland shows increased activity either with or before the onset of metamorphosis, and the greatly increased thyroidal activity as metamorphosis proceeds (4). To further investigate this supposition, the tadpoles of *Bufo vulgaris formosus* and *Rana japonica* at hind limb-bud stage were treated with combinations of DL-thyroxine and DCA in these experiments. The animals were collected in the vicinity of Tokyo. Three separate series of experiments were carried. They yielded uniformly similar results.

Received May 27, 1957.

EXPERIMENTAL METHODS AND RESULTS

1. *Effect of DCA on morphologic changes at metamorphosis induced by thyroxine.* On may 6, 26 tadpoles of *Bufo* with hind limbs of about 1.2 mm. in length were divided into six groups and observations were made over the following eight days. From that day, each group of tadpoles was kept in a finger bowl of 10 cm. diameter, containing 200 cc. of one of the following culture solutions: (a) 1.7×10^{-6} *M* DCA, (b) 3.4×10^{-6} *M* DCA, (c) 1.7×10^{-6} *M* DCA plus 5×10^{-8} *M* DL-thyroxine, (d) 3.4×10^{-6} *M* DCA plus

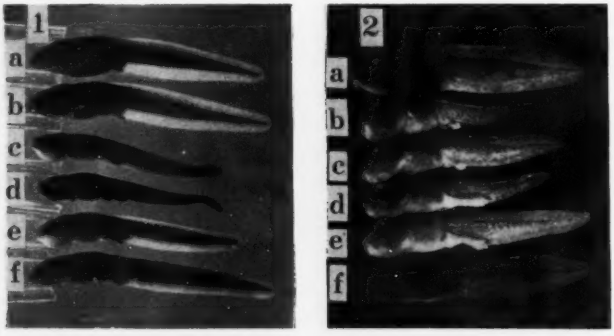


FIG. 1. Effect of thyroxine and DCA on metamorphosis of the tadpoles of *Bufo vulgaris formosus*. Tadpoles (a–f) were reared in following solutions, respectively: (a) 1.7×10^{-6} *M* DCA; (b), 3.4×10^{-6} *M* DCA; (c), 1.7×10^{-6} *M* DCA plus 5×10^{-8} *M* DL-thyroxine; (d), 3.4×10^{-6} *M* DCA plus 5×10^{-8} *M* DL-thyroxine; (e), 5×10^{-8} *M* DL-thyroxine; (f), tap water. ×1.5.

FIG. 2. Effect of implantation of pellets of thyroxine or DCA on metamorphosis of the tadpoles of *Rana japonica*. A representative tadpole from each group is shown. (a), Tadpole reared in DCA solution; (b), Tadpole reared in DCA solution and implanted with thyroxine pellet; (c), Tadpole reared in thyroxine solution and implanted with DCA pellet; (d), Tadpole reared in tap water and implanted with thyroxine pellet, (e), Tadpole reared in thyroxine solution, (f), Tadpole reared in tap water. Concentrations of the DCA and thyroxine solutions were 6.7×10^{-6} *M* and 12.5×10^{-8} *M*, respectively. ×1.1.

5×10^{-8} *M* DL-thyroxine, (e) 5×10^{-8} *M* DL-thyroxine, (f) tap water (control). These solutions were prepared by following methods, at least three days before use. The steroid was dissolved in the minimum volume of absolute alcohol and then diluted with tap water. Thyroxine was dissolved with slightly alkaline tap water. Control medium was adjusted to the same alkalinity. The molarity of the hormones is expressed in final concentration. During the experimental period culture solutions were not changed and no food was given the tadpoles. The temperature was about 18° C.

The photograph of a representative tadpole from each group on May 14, at the end of the period of study, is shown in Figure 1, and the details of observed metamorphic changes are presented in Table 1, and the results are summarized in Table 2. Metamorphosis was completely inhibited in the tadpoles kept in DCA, and they remained almost unchanged throughout the experiment. The forelegs and hind limbs of control tadpoles grew considerably. Thyroxine accelerated both the growth of forelegs and hind

TABLE 1. EFFECT OF DCA ON THYROXINE-INDUCED METAMORPHOSIS IN THE TADPOLES OF *Bufo vulgaris formosus*

(Numbers with standard errors are given in mm.)

Treatment from May 6 to May 14	No. of tadpoles	Length of trunk	Length of tail	Height of tail	Length of left hind limb	Length of left foreleg	Opercular perforation	Emergence of forelegs
$1.7\times10^{-6}M$ DCA	3	8.5±0.47	14.8±1.17	3.5±0.29	1.3±0.09[b,c]		−	−
$3.4\times10^{-6}M$ DCA	5	7.6±0.36	15.2±0.34[d]	3.7±0.09	1.4±0.05[a,c]		−	−
$5\times10^{-8}M$ Thyroxine plus $1.7\times10^{-6}M$ DCA	5	7.2±0.12[b]	13.2±0.61[b]	2.5±0.08[d]	3.1±0.05[c]	2.3±0.07[d]	+	+
$5\times10^{-8}M$ Thyroxine plus $3.4\times10^{-6}M$ DCA	5	6.9±0.17[b]	11.5±0.21[a,c]	2.0±0.18[c]	3.4±0.17[b]	2.5±0.15[a]	+	+
$5\times10^{-8}M$ Thyroxine	4	7.4±0.13	13.9±0.24[b]	2.9±0.12	3.8±0.08[a]	2.7±0.06[a,b]	−	−
Tap water (control)	4	7.8±0.20	16.3±0.73	3.3±0.17	2.4±0.25	1.7±0.02*	−	−

[a] $P<0.01$ compared with control tadpoles.
[b] $P<0.05$ compared with control tadpoles.
[c] $P<0.01$ compared with thyroxine-tadpoles.
[d] $P<0.05$ compared with thyroxine-tadpoles.
* Measured after dissection of tadpoles.

limbs and shortening of the tail. Measurements of the length of forelegs were made by dissecting them from their bodies, because they did not protrude out of the bodies at the end of the experiment. Other metamorphic changes such as decrease in trunk length, and tail height (measured at the middle of tail), opercular perforation and emergence of forelegs from body were not accelerated significantly by the thyroxine concentrations used within the time of observation in these experiments. Combination of thyroxine and either $1.7\times10^{-6}M$ DCA or $3.4\times10^{-6}M$ DCA had a synergistic effect on the reduction of trunk length, tail height, opercular perforation and emergence of forelegs. It seems likely from Figure 1 that the decrease in tail height is chiefly due to resorption of the tail fin. An augmenting effect of DCA on thyroxine action in decreasing tail length was observed in $3.4\times10^{-6}M$, but not in $1.7\times10^{-6}M$ DCA. On the contrary, DCA seems to antagonize the growth promoting effect of thyroxine on forelegs and

TABLE 2. SUMMARY OF THE EFFECT OF DCA ON THYROXINE-INDUCED METAMORPHOSIS OF *Bufo* TADPOLES AT HIND LIMB-BUD STAGE (8 DAYS AFTER TREATMENTS)

	Control	Treatment		
		Thyroxine	Thyroxine+DCA	DCA
Growth of forelegs	normal growth	acceleration	antagonizes action of thyroxine[1]	(Complete inhibition of all metamorphic changes)
Growth of hind limbs	normal growth	acceleration	antagonizes action of thyroxine[1]	
Emergence of forelegs	none	none	acceleration	
Shortening of tail	not observed	acceleration	augments action of thyroxine[2]	
Decrease in tail height	not observed	no significant acceleration	acceleration	
Shortening of trunk	not observed	no significant acceleration	acceleration	
Opercular perforation	none	none	acceleration	
Loss of teeth	half	complete	complete	
Shape of mouth	larval	larval	adult; acceleration	
Metamorphic dehydration*	normal loss of water	acceleration	augments action of thyroxine	

* Observation on dehydration was done in separate experiment.
[1] Effective dose, $1.7\times10^{-6}M$ DCA.
[2] Effective dose, $3.4\times10^{-6}M$ DCA.

hind limbs. In tadpoles given thyroxine and DCA, larval teeth disappeared and the shape of the mouth was almost of adult type. Thyroxine-tadpoles shed their teeth, but their mouths retained their larval shape. In control tadpoles about half of their teeth disappeared, but the mouths were still of larval type. In DCA-tadpoles teeth were all present and the mouths remained absolutely unchanged.

From the results thus obtained, it may be concluded that DCA given alone completely inhibits metamorphosis of tadpoles at hind limb-bud stage, but when thyroxine and DCA are given simultaneously, the steroid augments or antagonizes the effect of thyroxine depending upon the sites of metamorphic changes in the body. It would appear that DCA reinforces those metamorphic actions of thyroxine that are of an involutional nature (decreases in length and height of tail), but counteracts those actions which are revealed in growth (e.g. hind limbs and forelegs).

Repeating the same experiment with tadpoles of *Rana japonica* at hind limb-bud stage, similar results were obtained. When *Rana japonica* tadpoles reared in DCA solution were implanted with pellets of thyroxine, or thyroxine-solution reared tadpoles implanted with DCA pellets, the effects resembled those following use of the same hormones in solution. In the implantation experiment, the weights of pellets of thyroxine and DCA were about 0.05–0.1 mg. and about 0.1 mg., respectively. Experimental culture solutions made of tap water were $12.5\times10^{-8}M$ DL-thyroxine and $6.7\times10^{-6}M$ DCA. The pellets were implanted into the tail muscle near trunk. The temperature during these observations was about 25°C. It may be seen from Figure 2 that DCA completely inhibits metamorphosis in this species and thyroxine induces it; furthermore, DCA modifies the action of exogenous thyroxine in the manner seen in *Bufo* in the previous experiment.

By keeping them in progesterone, estrone, testosterone propionate, or cholesterol of 8 graded concentrations between $2.3\times10^{-7}M$ and $3.0\times10^{-5}M$, respectively, and also by keeping them in cortisone acetate of 3 different concentrations from $1.5\times10^{-6}M$ to $6.2\times10^{-6}M$, no clear effect on metamorphosis was observed in the absence of thyroxine in *Bufo* tadpoles at the two developmental stages mentioned above. Of these steroids, only testosterone propionate of $3.0\times10^{-5}M$ had a slight action, a relatively small delay in metamorphosis.

Anesthesia was produced by concentrations above $4.0\times10^{-6}M$ of progesterone and above $6.7\times10^{-6}M$ of DCA.

2. *Effect of DCA on dehydration at metamorphosis induced by thyroxine.* It is well established that water loss takes place at the time of metamorphosis of anuran tadpoles (5). In this experiment, a study was made of the effect of DCA on dehydration at the time of metamorphosis provoked by thyroxine in *Bufo* tadpoles. Experiments and observations were made during the period between June 3 and June 11. The final concentrations of DCA and thyroxine were 3.4×10^{-6} M and 5×10^{-8} M, respectively. The cultu-

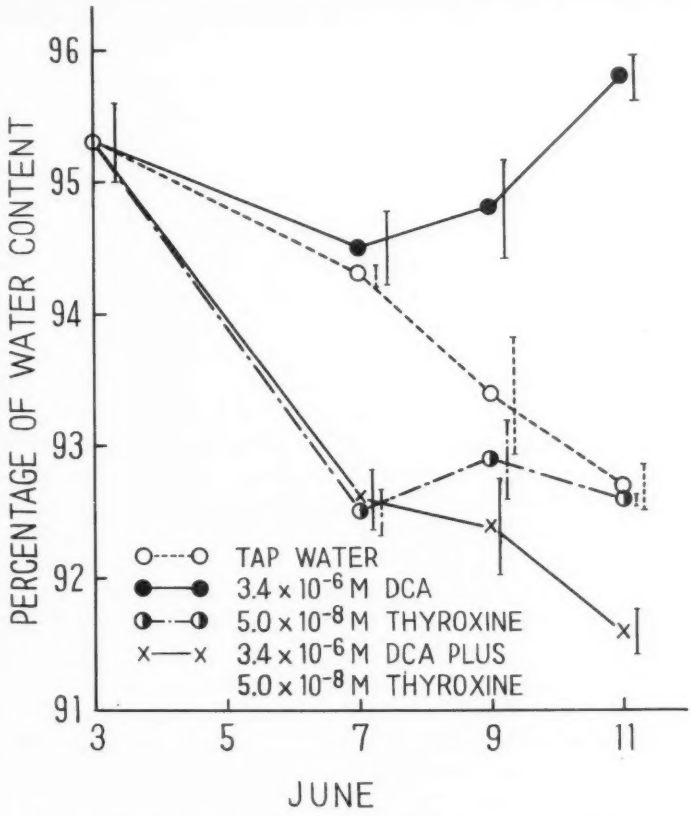


FIG. 3. Curves showing changes in the percentage ratio of the weight of body water to the weight of the body in experimental and control tadpoles of *Bufo vulgaris formosus*. Each value represents the mean of six tadpoles of each group. The vertical lines show the standard errors.

solutions were prepared by the same methods mentioned in the previous section. Toad tadpoles with hind limbs of about 1 mm. in length were employed and were kept without food in experimental and control solutions of 200 cc. listed in Figure 3. The animals were starved for five days before the beginning of the experiment to remove the intestinal contents. Temperature was kept at about 24° C throughout the experimental period. At intervals, wet and dry weights of six tadpoles of each group were determined to calculate the water fraction of the body.

The data are shown in Figure 3. Relative weight of body water decreased significantly ($P < 0.01$) in control tadpoles at the end of the experiment. DCA completely inhibited this water loss. In thyroxine-tadpoles significant dehydration ($P < 0.01$) was already recognized 4 days after the beginning of the experiment. On June 11, both thyroxine and control tadpoles showed

almost the same water content. The water loss in control tadpoles may be due to the thyroid hormone secreted by thryoid gland during normal metamorphosis. The difference in content of body water between the tadpoles treated with thyroxine plus DCA and thyroxine alone was significant ($P < 0.05$) on June 11. Thus DCA and thyroxine had a synergistic effect on dehydration at the time of metamorphosis.

DISCUSSION

In these experiments, the supposition was confirmed that the contradictory effect of DCA on metamorphosis may be associated with different levels of thyroid hormone in the body at the two developmental stages studied. The inhibiting action of DCA on metamorphosis of younger tadpoles (at hind limb-bud stage) may be due to small amounts of thyroid hormone in the body, since DCA accelerated metamorphosis when thyroxine was simultaneously added to the tadpoles at this developmental stage. And the augmenting effect of DCA on metamorphosis of tadpoles with 3 mm. hind limbs may result from the presence of adequate amounts of endogenous thyroid hormone in the body. Furthermore, it appears that DCA inhibits the functional development of the thyroid gland in tadpoles at hind limb-bud stage, since in these tadpoles metamorphosis is completely inhibited by DCA. If functional development of the thyroid had taken place normally in these animals, metamorphosis presumably would have occurred earlier than in the case of control tadpoles. Combinations of DCA and thyroxine had synergistic effects on emergence of forelegs, shrinking of tail, shortening of trunk, opercular perforation and transformation of mouth from larval shape into adult one. Earlier protrusion of fore legs in the tadpoles treated with DCA plus thyroxine is probably due to the precocious opercular perforation in these animals. On the other hand, DCA slightly, but significantly, inhibited the growth of forelegs and hind limbs during stimulation by thyroxine. These data suggest to us that DCA accelerates the degenerative changes of thyroxine-induced metamorphosis, but inhibits anabolic growth changes to a certain extent. Transformation of mouth shape from larval to adult type may be an exception to this generalization.

DCA caused tadpoles to retain body water when thyroxine was absent from the culture water, but it induced marked dehydration in the body when exogenous thyroxine was present. This suggests the co-operation of thyroxine and mineralocorticoids in the water loss at the time of anuran metamorphosis. Also in mammals, it has been shown that increased diuretic response to water by hyperthyroidism is largely abolished by adrenalectomy (6). On the other hand, it would seem that the prophlogistic activity of DCA described by Selye (7) could well be responsible for the acceleration of metamorphosis, since Aleschin (8) has shown that the histological changes during tail resorption present the characteristics of an inflamma-

tory reaction. At any rate, participation of mineralocorticoids in normal anuran metamorphosis is highly probable from the observation made by Stenger and Charipper (9) that marked changes take place in adrenal histology during metamorphosis.

Frieden and Naile (10) have observed acceleration of thyroxine ($5\times10^{-7}M$)-induced metamorphosis of *Bufo bufo bufo* by glucocorticoids, estrone and stilbesterol, but reported no effect of desoxycorticosterone ($2\times10^{-5}M$). This disagreement with the present data may be due chiefly to different concentrations of thyroxine and DCA employed. The author observed in some experiments that high concentrations of DCA such as $7\times10^{-6}M$ inhibited resorption of the tail induced by $5\times10^{-8}M$ thyroxine. This, and the other data now available, suggest that the ratio of thyroxine and DCA is important in affecting the metamorphosis of tadpoles. Bock (11) and Gasche (3) reported an increase in metamorphic rate of thyroxine-treated tadpoles by using cortin and DCA, respectively. The dosage used by them apparently was adequate to accelerate metamorphosis. The failure of estrone and cortisone acetate to affect metamorphosis in the present experiment possibly may also be ascribed to inadequate concentrations of the hormones for the tadpoles of *Bufo vulgaris formosus*. Negative effects of cortisone on metamorphosis also have been reported by other investigators (12, 13).

Acknowledgments

I wish to thank Professor K. Takewaki for his encouragement, and also Drs. C. D. Turner and A. Gorbman for their kind help in preparing the manuscript.

REFERENCES

1. Kobayashi, H. and K. Okubo: *Annot. Zool. Japon.* **27:** 173. 1954.
2. Okubo, K.: *Zool. Mag.* **63:** 99. 1955.
3. Gasche, P.: *Helv. Physiol. Phamacol. Acta* **3:** C 10. 1945.
4. Lynn, W. G. and H. E. Wachowski: *Quart. Rev. Biol.* **26:** 123. 1951.
5. Etkin, W.: *Physiol. Zool.* **5:** 275. 1932.
6. Gaunt, R., M. Cordsen and M. Liling: *Endocrinology* **35:** 105. 1944.
7. Selye, H.: *The story of the adaptation syndrome*, Acta Inc., Med. Pub., Montreal. 1952.
8. Aleschin, B.: *Bull. Histol.* appl. **12:** 5. 1935.
9. Stenger, A. and H. A. Charipper: *J. Morph.* **78:** 27. 1946.
10. Frieden, E. and B. Naile: *Science* **121:** 37. 1955.
11. Sluczewski, A. and P. Roth: *Ann. endocrinol.* **14:** 948. 1953.
12. Kuusisto, A. N. and Telkkä: *Acta Endocrinol.* **13:** 61. 1953.
13. Bock, K. A.: *Klin. Wochschr.* **17:** 1311. 1938.

ENHANCED DESOXYRIBONUCLEIC ACID ACCUMULATION AND RETARDED PROTEIN ACCUMULATION IN SKELETAL MUSCLE OF "HYPOPHYSECTOMIZED" CHICK EMBRYOS[1]

DAVID S. LOVE AND IRWIN R. KONIGSBERG

Division of Chemical Embryology, University of Colorado, School of Medicine, Denver, Colorado

ABSTRACT

Hormonal regulation of the chemical composition of developing skeletal muscle was examined by the use of chick embryos in which the prosencephalon was surgically removed at Hamburger-Hamilton stages 10 to 12. Control and operated animals were sacrificed at two-day intervals from the 10th through the 20th day of development and homogenates of leg muscle prepared and assayed for DNA, RNA and protein nitrogen content.

Muscle fresh weight in the experimental group is normal on the sixteenth day but thereafter increases at a significantly slower rate than in the control group.

On the 16th day of development both the RNA and protein nitrogen content of the leg muscle in the experimental embryos are significantly lower than the control levels. However, although the differences in protein nitrogen content in the two groups becomes progressively larger the initially observed differences in RNA levels is maintained throughout the remainder of the experimental period.

In contrast to the other growth parameters studied DNA accumulation is enhanced in the absence of the derivatives of the prosencephalon. DNA accumulation normally decreases sharply after the 16th day of development. In the experimental group rapid accumulation continues for at least another 48 hours. Consequently, in the operated embryos, the DNA content of the leg musculature is 16% and 22% greater at 18 and 20 days respectively than in control embryos.

THE limb bud makes its appearance during the 3rd day of the development of the chick embryo (1), and arises, according to Strauss and Rawles (2), from the mesenchymal tissue of the lateral plate mesoderm. Although rudimentary function has been demonstrated as early as the 6th day (3), rapid accumulation of contractile proteins does not begin until the 11th day (4). The structural protein, collagen, has also been shown to increase rapidly at the same time (5). Inasmuch as these events occur in histologically different cell types (myoblasts and fibroblasts, respectively) there is a suggestion of the appearance of one or more general regulatory mechanisms governing protein accumulation during this period. Becaus

Received July 5, 1957.

[1] Supported by grants from the National Science Foundation and the U. S. Public Health Service (Grant #B-549).

of the general growth regulating effects of the endocrine system, it seemed reasonable to investigate the possibility of hormonal influences on leg muscle development by extirpating the pituitary primordia.

In addition to weight measurements, accumulation of protein nitrogen (PN) was used to determine cell growth. Deoxyribonucleic acid (DNA) was used as an index of cell proliferation on the basis of the experiments of Davidson and Leslie (6, 7) in which the constancy of this material per nucleus was demonstrated. In view of the recent evidence indicating the involvement of ribonucleic acid (RNA) in protein synthesis (8) this component of embryonic muscle was measured also.

MATERIALS AND METHODS

Fertile eggs of the Hy-Line strain of a White Leghorn-Delaware hybrid fowl were incubated in a forced-draft incubator maintained at 37.5° C. These eggs were opened after 33–40 hours by sawing a small window over the blastodisc, the position of which was determined by candling. Embryos were hypophysectomized at Hamburger-Hamilton (9) stages 10 to 12 by decapitation at the level of the middle of the mesencephelon, thus removing completely both primordia of the pituitary gland (10). The eggs were sealed by replacing the shell fragment and securing it in place with molten paraffin. The same procedure was followed for the controls with exception of decapitation.

Approximately 50% of the hypophysectomized embryos survived to the 14th day and about half of these reached the 20th day of development. These and the control embryos were sacrificed at two-day intervals from the 12th to the 20th day by severing the remaining portion of the head of the experimentals and the head of the controls at the level of the base of the mandible and the second cervical vertebra. The carcasses were blotted on paper toweling and the body weight, minus head, was determined. The leg muscle was dissected according to the method described by Konigsberg and Herrmann (11) but was quickly weighed on a Roller-Smith torsion balance before freezing to avoid any error in weighing which might result from moisture condensation onto the frozen muscle.

The frozen samples were homogenized in a Potter glass homogenizer immersed in an ice bath using an equal volume of cold normal saline solution for the 12, 14, and 16-day embryos and twice this volume for the 18 and 20-day embryos. Following homogenization these samples were diluted to contain 100 mg./ml. with the exception of the 12-day sample which was diluted to 50 mg./ml. to provide an ample volume for analysis.

Duplicate aliquots of 0.25 ml. for PN determination and 1.0 ml. for nucleic acid determination were pipetted into 12 ml. graduated centrifuge tubes. All samples were washed with cold 5% TCA and carried through a lipid extraction with 50% ether-alcohol at 47° C. The PN samples were digested in sulphuric acid containing a selenium catalyst, while the samples reserved for nucleic acid determination were extracted with hot TCA according to the method of Schneider (12). The diphenylamine method of Dische (13) was used for the colorimetric assay of DNA, and the method of Euler and Hahn (14) for RNA. The color densities were measured in a Beckman model DU spectrophotometer at wave lengths of 620 lambda for RNA and 600 lambda for DNA. The digested PN samples were nesslerized and read at 500 lambda in a Coleman colorimeter.

RESULTS

As shown in Table 1, body weight augmentation is not affected by decapitation until the 16th day of development, when a statistically signifi-

TABLE 1. BODY WEIGHT (MINUS HEAD) OF CONTROL AND DECAPITATED EMBRYOS

Days of development	Decapitates I		Controls II		Mp I and II
	No. of cases	Mean ±S.E., gm.	No. of cases	Mean ±S.E., gm.	
12	16	2.09±0.15	14	2.11±0.10	0.9
14	13	4.25±0.28	13	4.97±0.45	0.2
16	8	6.22±0.28	9	8.40±0.45	0.001
18	16	9.18±0.30	13	14.51±0.62	0.001
20	14	10.15±0.37	8	18.51±1.37	0.001

Mp—The level of confidence in the difference between the means of the decapitates (I) and the controls (II).
S.E.—Standard error of the mean.

cant difference of 37% becomes apparent between the body weights of control and experimental embryos. By the 18th day this has increased to 63% and is maintained (58%) on the 20th day. Inhibition of fresh muscle weight increase (Table 2), on the other hand, does not appear until the 18th day when the weight of the experimental embryo's leg muscle is 18% less than the controls'. This difference increases to 33% by the 20th day.

The marked increase in body weight difference from the 16th to the 18th day is the result of a sharp rise in the rate of body weight augmentation in the control embryo during the 16 to 18-day interval. This is shown to be significant by the calculation and statistical comparison of the regression coefficient for the 14 to 16-day interval to that for the 16 to 18-day period (1.583 gm./day to 3.095 gm./day, $P < 0.001$). No significant change in the rate of body weight increase of the experimental embryos was observed during the entire 12 to 20-day interval. The differences in fresh muscle weight, however, are due to a significant reduction in the rate of muscle weight augmentation in the decapitated embryos during the 16 to 20-day period compared to that from 12 to 16 days (110 mg./day to 30.4 mg./day, $P < 0.001$). There is no variation in the rate of fresh muscle weight augmentation in the normal embryos from the 12th to the 20th day of development.

From the data presented in Table 3 it can be seen that no significant differences in DNA content can be demonstrated prior to the 16th day.

TABLE 2. FRESH MUSCLE WEIGHT OF ONE LEG IN CONTROL AND DECAPITATED EMBRYOS

Days of development	Decapitates I		Controls II		Mp* I and II
	No. of cases	Mean ±S.E., mg.	No. of cases	Mean ±S.E., mg.	
12	10	63.3± 7.9	9	98.8± 9.9	0.5
14	16	239.0±11.0	16	261.0±18.0	0.3
16	22	454.0±19.0	19	457.0±19.0	0.9
18	19	560.0±21.0	15	684.0±31.0	0.01
20	18	618.0±26.0	13	925.0±57.0	0.001

* Mp—The same as in Table 1.

TABLE 3. DEOXYRIBONUCLEIC ACID CONTENT OF THE LEG MUSCULATURE

Days of development	Decapitates I		Controls II		Mp* I and II
	No. of cases	Mean ±S.E., mg.	No. of cases	Mean ±S.E., mg.	
12	10	0.204 ±0.018	9	0.221 ±0.031	0.6
14	9	0.575 ±0.017	8	0.642 ±0.058	0.9
16	9	0.917 ±0.053	8	1.150 ±0.084	0.8
18	11	1.494 ±0.074	8	1.274 ±0.042	0.02
20	9	1.676 ±0.083	6	1.302 ±0.058	0.01

* Mp—The same as in Table 1.

After this time, however, the DNA values of the experimental group are significantly higher than those of the controls, and are the result of a marked drop in the rate of DNA accumulation in the controls after the sixteenth day. (No significant differences exist between the DNA levels of the control embryos at 16, 18 and 20 days.) In contrast to this, DNA accumulation in the experimental embryos continues at a considerable rate until the 18th day and only thereafter begins to decline.

The first significant difference between experimental and control embryos in both PN (Table 4) and RNA (Table 5) content of the leg musculature occurs on the 16th day of development and is the result of an increased rate of accumulation of both components in the control embryos during the 14 to 16-day interval with no corresponding increase in the experimental embryos. A small but statistically significant increase in the rate of PN accumulation in the experimental group was observed during the 16 to 18-day interval, but the PN level of the leg musculature of the experimental embryos never reaches the level attained by the control group. The rate of RNA accumulation in the experimental embryos remains the same over the entire 12 to 20-day period. The initial difference in RNA content between controls and experimentals, established on the 16th day, is maintained through the 20th day.

DISCUSSION

The exclusion of the pituitary from the developing embryo results in the prolongation of the period during which rapid accumulation of DNA

TABLE 4. PROTEIN NITROGEN CONTENT OF THE LEG MUSCULATURE

Days of development	Decapitates I		Controls II		Mp* I and II
	No. of cases	Mean ±S.E., mg.	No. of cases	Mean ±S.E., mg.	
12	10	0.621 ±0.071	9	0.886 ±0.217	0.2
14	9	1.909 ±0.049	9	2.401 ±0.335	0.1
16	9	3.597 ±0.236	8	5.720 ±0.612	0.01
18	11	6.198 ±0.427	7	9.272 ±0.724	0.001
20	9	8.331 ±0.500	6	14.411 ±1.153	0.001

* Mp—The same as in Table 1.

occurs in the leg musculature. By the 18th and 20th day of development the operated embryos exhibit strikingly greater DNA per organ levels than their normal controls. The higher DNA levels in the experimental animals may represent either continued nuclear proliferation or the attainment of polyploidy by a portion of the nuclear population. An increase in diploid nuclei concomitant with a decrease in the number of tetraploid nuclei, has in fact, been observed in the liver of hypophysectomized adult rats (15). A shift in the opposite direction, however, would be required to explain

TABLE 5. RIBONUCLEIC ACID CONTENT OF THE LEG MUSCULATURE

Days of development	Decapitates I		Controls II		Mp* I and II
	No. of cases	Mean ±S.E., mg.	No. of cases	Mean ±S.E., mg.	
12	8	0.332±0.018	7	0.315±0.041	0.8
14	9	0.922±0.061	8	1.113±0.119	0.1
16	9	1.463±0.091	8	2.210±0.211	0.01
18	9	2.068±0.139	6	2.874±0.208	0.01
20	9	2.486±0.144	6	3.321±0.265	0.02

* Mp—The same as in Table 1.

the observed increases in DNA in the present study, assuming that such shifts are not restricted to organs normally exhibiting a high degree of polyploidy. Irrespective of the cytological distribution of the additional DNA, the data suggest that in normal development the synthesis of this component of muscle cells is curtailed by a hormonally regulated mechanism.

In contrast to the DNA content both the protein nitrogen and RNA values for the total leg musculature of the operated embryos are significantly lower than the control levels. Moreover these differences develop concomitantly which in the light of current thought on the role of RNA in protein synthesis (8), is rather suggestive. The decrease in PN and RNA accumulation by decapitation indicates that the hypophysis is involved in the rapid accumulation of leg muscle protein that begins between the 14th and 16th day.

The differences between experimental and control values for protein nitrogen and RNA per total leg musculature observed in the present study are closely similar to those reported for thiourea-treated embryos (16). The present results differ from those observed in thiourea-treatment, however, in that the continued accumulation of DNA is much more marked. Treatment with the goitrogen resulted in a more rapid accumulation of DNA from the 16th to the 18th day but the DNA content never exceeded the control level as observed in the present study. The results reported, therefore, cannot be ascribed entirely to hypothyroidism.

In view of the central role of the pituitary in endocrine regulation, it is quite possible that the observed effects of the absence of the pituitary are

mediated through dependent endocrine organs. Several endocrine effects on cell proliferation have been described. For example, Stevens and Reid (17) report a large increase in desoxyribonuclease activity in the liver cells of adrenalectomized rats accompanied by an increase in DNA content, but only a small increase in DNAase activity without an accompanying increase in DNA content occurs as a result of hypophysectomy. Since a marked inhibition of the development of the adrenal cortex of the chick embryo has been shown by Case (18) to be a consequence of decapitation, the possibility exists that continued DNA accumulation in the decapitates is due to disturbed adrenal function and not directly to the absence of hypophyseal hormones.

Although it is not possible at this time to attribute the results of decapitation to specific hormones, the similarity between the decapitated chick embryos and hypophysectomized (19), thyroidectomized (20) and doubly operated (21) neo-natal rats suggests a synergistic action of growth hormone and thyroxine on cell growth. Scow (20) has shown that the retardation of myosin and collagen accumulation in either hypophysectomized or thyroidectomized neo-natal rats cannot be completely reversed by supplementing growth hormone alone. Konigsberg (16), on the other hand, was able to show complete reversal of the effects of thiourea treatment on PN and RNA accumulation by administration of thyroxine. In contrast to this, Jost has reported that body weight augmentation of decapitated, thyroidectomized, or doubly operated rabbit fetuses is not significantly impaired (22). But the effects of these operations are complicated by the presence of maternal hormones and Jost points out that extirpation of the maternal endocrine organs in addition to those of the fetus is necessary before the results can be completely conclusive.

The discrepancy between the time of inhibition of muscle weight increase and accumulation of muscle components might be the result of disturbed water balance, caused by the removal of the posterior pituitary primordia, and resulting in fluid retention in the muscle.

Acknowledgments

The authors gratefully acknowledge the suggestions and advice of Dr. Heinz Herrmann during the course of this investigation.

REFERENCES

1. Hamilton, H. L.: Lillie's Development of the Chick. Henry Holt and Co., New York. pp. 540. 1952.
2. Rawles, M. E. and W. L. Strauss, Jr.: *Anat. Rec.* **100**: 755. 1948.
3. Kuo, Z. Y.: *Jour. Exp. Zool.* **32**: 371. 1939.
4. Csapo, A. and H. Herrmann: *Am. Jour. Physiol.* **165**: 701. 1951.
5. Herrmann, H. and S. R. Barry: *Arch. Biochem. & Biophys.* **55**: 526. 1955.
6. Davidson, J. N. and I. Leslie: *Biochem. Jour.* **46**: xl. 1950.
7. Herrmann, H., B. N. White and M. Cooper: *Jour. Cell. & Comp. Physiol.* **49**: 227. 1957.

8. Spiegelman, S.: In: Symposium on the Chemical Basis of Heredity. Ed.: W. D. McElroy and H. B. Glass. Johns Hopkins Press, Baltimore. p. 232. 1957.
9. Hamburger, V. and H. L. Hamilton: *Jour. Morph.* **88:** 49. 1951.
10. Fugo, N. W.: *Jour. Exp. Zool.* **85:** 271. 1940.
11. Konigsberg, I. R. and H. Herrmann: *Arch. Biochem. & Biophys.* **55:** 534. 1955.
12. Schneider, W. C.: *Jour. Biol. Chem.* **161:** 293. 1945.
13. Dische, Z.: *Mikrochemie* **8:** 4. 1930.
14. Euler, H. V. and L. Hahn: *Svensk. Kem. Tidskv.* **58:** 251. 1946.
15. Di Stefano, H. S., H. F. Diermeier and J. Tepperman: *Endocrinology* **57:** 158. 1955.
16. Konigsberg, I. R.: *Jour. Cell. & Comp. Physiol.* In press. 1958.
17. Stevens, B. M. and E. Reid: *Biochem. Jour.* **64:** 735. 1956.
18. Case, J. F.: *Ann. N. Y. Acad. Sci.* **55:** 147. 1952.
19. Scow, R. O.: *Endocrinology* **55:** 44. 1954.
20. Scow, R. O.: *Am. Jour. Phys.* **173:** 199. 1953.
21. Simpson, M. E., C. W. Asling and H. M. Evans: *Yale Jour. Biol. Med.* **23:** 1. 1950.
22. Jost, A. and L. Picon: Symp. Soc. Exptl. Biol. XI. Academic Press, New York. p. 228, 1957.

HORMONAL BALANCE AND LIVER CATALASE ACTIVITY[1]

ROBERT C. TROOP

Division of Pharmacology, University of Tennessee Medical Units, Memphis, Tennessee

ABSTRACT

Adrenocortical steroids and various dietary factors were studied for their effects on liver catalase activity. Rats which were force fed a nutritionally adequate diet showed reduced liver catalase activity; this reduction was not influenced by corticosterone administration. Animals fed *ad libitum* responded to corticosterone with a slight decrease in catalase activity. Rats denied food for 5 days showed diminished catalase activity and this effect was greatly accentuated by giving cortisone acetate during the fasting period. The ability of hydrocortisone acetate to suppress catalase activity was abolished when desoxycorticosterone acetate (DCA) was administered concurrently. DCA, by itself, had no effect on this enzyme. A tentative hypothesis relating to the hormonal control of catalase activity is proposed. It is based on the premise that the adrenocortical secretions are the principal hormonal factors in catalase regulation. The modifications in catalase activity seen after other endocrine changes are, according to this hypothesis, due to simultaneous quantitative or qualitative changes in adrenocortical secretion.

VARIOUS modifications of adrenocortical function have been shown to influence liver catalase activity in the rat (1, 2). Cortisone and hydrocortisone produce a reduction of catalase activity; adrenalectomy or administration of ACTH, however, do not affect the activity of this enzyme. This interesting discrepancy has prompted further investigation of the role of the adrenal cortex in catalase regulation.

Four steroids now known to be secreted by the isolated adrenal of normal rats are corticosterone, hydrocortisone, cortisone and aldosterone (3, 4). These steroids are normally secreted in rather fixed proportions but the rate of secretion of the individual hormones can vary independently and several factors are known which modify the ratio of one to another (5, 6, 7). As suggested previously (1), the explanation for the lack of an effect with ACTH on catalase activity may lie in the concept of relative proportions of circulating adrenal cortical steroids. There is evidence (8) that athough ACTH increases the total secretion of the adrenal cortex, no change in the relative amounts of the various steroids occurs in short term experiments. However, the injection of exogenous steroids would seriously upset the

Received July 30, 1957.

[1] This investigation was supported in part by U. S. Public Health Service grant No. C-3191. Part of this work was done at the University of Oklahoma School of Medicine.

overall pattern. If this concept has basis in fact, it should be possible to administer adrenal steroids in proportions which do not affect liver catalase activity. This paper gives the results of investigations on this and related problems.

METHODS

Male rats of the Holtzman strain were used throughout these experiments. They were maintained on Purina laboratory chow and water *ad libitum* unless otherwise stated.

Catalase activity of liver homogenates was measured by the sodium perborate method (9) and activity is expressed as milliequivalents of sodium perborate destroyed per gram of liver or per milligram of nitrogen. Details of this procedure are given in the previous study (1).

Hydrocortisone was administered by intragastric intubation as the acetate in saline suspension. Desoxycorticosterone acetate (DCA) and cortisone acetate were given subcutaneously in saline suspension. Corticosterone was given subcutaneously in sesame oil.

The semiliquid diet used for force feeding in this investigation furnished each rat daily 47 calories, 3.2 gm. protein, 1.3 gm. fat, 5.6 gm. carbohydrate, vitamins and minerals in nutritionally adequate quantities. Each rat was fed morning and evening during the five day experimental period. The animals were adjusted gradually to the diet by giving increasingly larger amounts for several days before they were maintained entirely on this diet. During the period of adjustment they had access to their regular diet of laboratory chow. The *ad libitum* fed control animals were handled in a similar way in that the feeding tube was passed each time the force fed animals received it but no food was introduced.

The adrenal glands and spleen respond with characteristic weight changes to many endocrine imbalances. For this reason, adrenal and spleen weights are recorded. The adrenal glands were dissected free from fat and connective tissue and weighed individually to the nearest 0.1 mg. Tabulated values represent the average weight for a single gland.

Total nitrogen content of the liver homogenates was determined by a micro-Kjeldahl procedure.

RESULTS

Corticosterone appears to be the principal secretory product of the rat adrenal cortex (10). In view of this it was important to determine the effect of this steroid on catalase activity. The glucocorticoids are known to reduce food intake of experimental animals thereby introducing inanition as a possible causative factor in any study involving these steroids. With this in mind, corticosterone was first studied in rats force fed a semiliquid diet. The data in Table 1 indicate that the force feeding procedure by itself significantly decreased catalase activity. This is apparent when catalase activity is expressed either as m.eq./mg. nitrogen or m.eq./gm. liver. Because catalase assay results were independent of the manner of calculation, the statistical significance of the differences between groups is given only for the data calculated on the tissue weight basis. The reason force feeding should depress catalase is obscure but may be related to the stress

TABLE 1. EFFECT OF DIET AND STEROIDS ON LIVER CATALASE ACTIVITY IN MALE RATS. EIGHT RATS PER GROUP

Group No. and treatment	Body weight		Liver weight	Adrenal weight	Catalase activity*	
	Initial	Terminal			Per mg. N.	Per gm. liver†
	gm.	gm.	gm.	mg.		
1. Fed *ad libitum*	300 ± 11	299 ± 12	9.5 ± .8	20.7 ± 1.6	19.9 ± .33	656 ± 13
2. Force fed, 5 d.	297 ± 13	289 ± 14	9.2 ± .4	22.5 ± .7	16.8 ± .93	564 ± 25
3. Force fed plus corticosterone, 5 mg./d.; 5 d.	301 ± 14	281 ± 10	10.8 ± .4	17.6 ± .6	15.5 ± .65	553 ± 25
4. Fed *ad libitum*	375 ± 12	385 ± 12	9.7 ± .2	23.9 ± .5	26.0 ± 1.2	815 ± 31
5. Fasted, 5 d.	415 ± 9	372 ± 9	6.8 ± .1	24.5 ± .2	21.2 ± 1.3	721 ± 33
6. Fasted plus cortisone acetate, 5 mg./d.; 5 d.	419 ± 11	363 ± 9	9.0 ± .2	20.2 ± .8	17.7 ± .56	529 ± 27

All results in this and the following tables are given as Mean ± S.E.
* Milliequivalents perborate decomposed.
† Difference between group 1 and group 2: significant P = <.05
Difference between group 1 and group 3: significant P = .02
Difference between group 2 and group 3: not significant P = >.5
Difference between group 4 and group 5: significant P = .05
Difference between group 4 and group 6: significant P = <.01
Difference between group 5 and group 6: significant P = <.01

involved. This is supported by the somewhat larger adrenal glands of the force fed rats (Group 2) as compared to the ones fed *ad libitum* (Group 1). Corticosterone did not modify the catalase response to force feeding (Group 3). When administered to rats fed the regular cube diet, this steroid produced a slight reduction of catalase activity (Table 2, Group 2).

In view of the indication that force feeding decreases catalase activity it seemed of interest to reinvestigate the effect of cortisone on this enzyme under conditions which would not allow variations in food intake to be a factor. In the lower part of Table 1, it is shown that fasting for 5 days (rats allowed water only) produces a significant reduction of catalase activity and that cortisone acetate given during this period of fasting greatly intensifies the reduction. Miller (11) has found a 7-day fast to result in loss of rat liver catalase activity.

The animals in Table 2 were fed the regular cube diet *ad libitum*. The catalase-depressing property of cortisone acetate is not decreased by the simultaneous administration of corticosterone (Group 5).

Table 3 records results following hydrocortisone acetate and DCA administered individually and in combination. The animals in Group 1 were divided into two equal groups. One group received no treatment while

TABLE 2. EFFECT OF ADRENOCORTICAL HORMONES ON LIVER CATALASE ACTIVITY IN MALE RATS. EIGHT RATS PER GROUP

Group No. and treatment	Body weight		Liver weight	Spleen weight	Adrenal weight	Catalase activity*
	Initial	Terminal				
	gm.	gm.	gm.	mg.	mg.	
1. None	253 ± 13	258 ± 14	8.8 ± .6	657 ± 80	18.5 ± .6	822 ± 16
2. Corticosterone, 5 mg./d.; 5 d.	226 ± 9	219 ± 7	7.8 ± .4	399 ± 74	12.8 ± .6	702 ± 43
3. DCA, 5 mg./d.; 5 d.	258 ± 7	264 ± 11	9.4 ± .4	678 ± 77	16.3 ± .8	809 ± 32
4. Cortisone acetate, 5 mg./d.; 5 d.	255 ± 9	227 ± 9	—	—	13.5 ± .6	445 ± 20
5. Corticosterone, 5 mg./d.; 5 d. plus Cortisone acetate, 5 mg./d.; 5 d.	252 ± 9	219 ± 8	9.7 ± .3	322 ± 71	12.1 ± .3	404 ± 17

* Milliequivalents perborate decomposed per gm. liver.
Difference in catalase activity in group 1 and group 2: significant P = .05
Difference in catalase activity in group 1 and group 3: not significant P = >.5
Difference in catalase activity in group 1 and group 4: significant P = <.01

the rats in the other group were injected daily for 5 days with 0.2 cc of the vehicle in which hydrocortisone was suspended (Aqueous Vehicle #1, Merck). Data (body weight, organ weights, catalase activity) from the two groups did not differ and are therefore reported together. The hydrocortisone treated animals of Group 2 exhibit a reduced catalase activity in confirmation of our previous observation (1). However, when DCA, which by itself exerts no significant effect (Table 2, Group 3; Table 3, Group 3), is given concurrently with hydrocortisone acetate the action of the latter toward catalase activity is abolished (Group 4).

TABLE 3. EFFECT OF HYDROCORTISONE ACETATE AND DCA ON LIVER CATALASE ACTIVITY IN MALE RATS

Group No. and treatment	No. of rats	Body weight		Liver weight	Spleen weight	Adrenal weight	Catalase activity*
		Initial	Terminal				
		gm.	gm.	gm.	mg.	mg.	
1. None	12	375±6	395±6	12.6±.4	757±23	22.6±.4	829±19
2. Hydrocortisone acetate, 5 mg./d.; 5 d.	6	393±5	381±7	11.6±.2	626±29	19.2±.7	673±28
3. DCA, 2.5 mg./d.; 5 d.	7	387±7	399±7	11.4±.4	810±39	20.2±.5	878±18
4. DCA, 2.5 mg./d.; 5 d. plus hydrocortisone acetate, 5 mg./d.; 5 d.	7	394±7	393±7	11.6±.4	700±35	17.6±.5	806±22

* Milliequivalents perborate decomposed per gm. liver.
Difference in catalase activity in group 1 and group 2: significant $P = <.01$
Difference in catalase activity in group 1 and group 3: not significant $P = .1$
Difference in catalase activity in group 1 and group 4: not significant $P = >.5$

Gaebler and Mathies (12) have reported liver catalase activity to be increased 2 to 3 fold in female rats following hypophysectomy. Small doses of growth hormone which produced almost as much gain in weight as occurred in normal controls did not lower the high catalase level of the operated animals. Catalase activity was, however, restored to normal with doses of growth hormone twenty times as large as the dose effective in producing gain in body weight. We have had the opportunity to study catalase activity in male rats following hypophysectomy. The liver catalase activity of 9 rats 170 days following hypophysectomy was (871 m.eq.) significantly ($P < .05$) higher than in unoperated control animals (755 m.eq.). This agrees in general with Gaebler and Mathies but the magnitude of change is much less in our experiment. The time factor may account for the difference. Their animals were studied about 35 days following hypophysectomy.

DISCUSSION

The endocrine regulation of catalase activity is a complex mechanism and present knowledge is insufficient to allow a precise description to be made. Nonetheless, it is interesting to speculate whether or not the various hormonal factors studied have a common basis for their effect on catalase activity. After consideration of the available evidence we have made the tentative assumption that the adrenal steroids are the principal hormonal entities influencing the action of this enzyme in the rat. In this paper ev-

dence is presented which indicates that the ratio of mineralocorticoids to glucocorticoids may be of more importance in this respect than the absolute quantities of either.

On the assumption stated above, it follows that the increased catalase activity seen after hypophysectomy might be due to an imbalance of adrenocortical hormones. That a shift toward a preponderance of mineralocorticoids in the adrenal secretion of the rat occurs, following hypophysectomy, has been demonstrated by Singer and Stack-Dune (6).

Another line of evidence that fits this hypothesis is from the work of Begg *et al.* (2). They demonstrated a 20% reduction of liver catalase activity in male rats treated with diethylstilbestrol. It is known that estrogens cause stimulation of the adrenal cortex via an increased release of ACTH (13). There is also evidence that the pattern of adrenal cortical secretion in response to ACTH may be modified by sex hormones (7). In this indirect way the corticoids may be responsible for the catalase depression seen in estrogen treated rats. The lower catalase activity in female rats as compared to that of males (14, 15, 1) might be explained in a similar manner.

If the hypothesis that the adrenocortical hormones are the principal factors in catalase regulation is to hold, it must also explain the seemingly paradoxical situation wherein exogenous cortisone and hydrocortisone cause a marked reduction of catalase activity while administration of ACTH or adrenalectomy produce no observable change. Although ACTH administration increases the total secretion rate of adrenal steroids, the relative amounts of the various steroids may not change (8). The injection of corticoids does, however, abruptly modify the quantitative relationship between the various circulating adrenocortical steroids. From the data of the present investigation it appears that an over balancing of this relationship in favor of corticosterone or DCA has no serious effect on catalase but that a relative excess of hydrocortisone (or cortisone) profoundly depresses the activity of this enzyme and that this depression can be prevented with DCA but not with corticosterone. It is quite possible that ACTH fails to modify catalase activity because, under its influence, the various components of the adrenocortical secretion are increased in such a manner that a preponderance of catalase-depressing steroids does not occur.

The concept of hormonal balance as an important factor in the influence of hormones on enzymes is not new. The hormonal regulation of hexokinase is an outstanding example. Insulin on the one hand and growth hormone and 11-oxycorticosteroids on the other have a competitive action on this enzyme, the latter inhibiting the reaction catalyzed by hexokinase and the former overcoming this inhibition. Evidence is accumulating to indicate that the control of hexokinase activity is primarily dependent on the ratio of pituitary-adrenal to insulin activity, rather than on the absolute amounts of either hormone. In the absence of all three, carbohydrate utili-

zation (as an index of hexokinase activity) may be almost normal (16). This normalcy of enzyme function in the absence of the competitive forces may be the reason adrenalectomy fails to modify catalase activity. It is interesting to note here that cortisone produces a depression of catalase even in adrenalectomized rats (2).

Acknowledgments

The author is indebted to the following for materials used in this study: Dr. E. Henderson, Schering Corp. for desoxycorticosterone acetate; Dr. F. K. Heath, Merck and Co. for corticosterone; and Dr. Elmer Alpert, Merck and Co. for hydrocortisone acetate.

REFERENCES

1. Troop, R. C. and A. J. Stanley: *Arch. Biochem. and Biophys.* **60:** 419. 1956.
2. Begg, R. W., T. E. Dickinson and A. V. White: *Can. J. Med. Sci.* **31:** 307. 1953.
3. Heard, R. D. H., *et al.*: *Recent Progress in Hormone Research* **9:** 383. 1954.
4. Eisenstein, A. B.: *Proc. Soc. Exp. Biol. and Med.* **91:** 657. 1956.
5. Eisenstein, A. B. and P. M. Hartroft: *Endocrinology* **60:** 634. 1957.
6. Singer, B. and M. P. Stack-Dunne: *J. of Endocrinology* **12:** 130. 1955.
7. Macchi, I. A., *et al.*: *Physiol. Reviews* **34:** 491. 1954.
8. Kass, E. H., *et al.*: *Proc. Soc. Exp. Biol. and Med.* **85:** 583. 1954.
9. Feinstein, R. N.: *J. Biol. Chem.* **180:** 1197. 1949.
10. Bush, I. E.: *J. of Endocrinology* **9:** 95. 1953.
11. Miller, L. L.: *J. Biol. Chem.* **172:** 113. 1948.
12. Gaebler, O. H. and J. C. Mathies: *Endocrinology* **48:** 623. 1951.
13. Ingle, D. J. and B. L. Baker: *Endocrinology* **48:** 764. 1941.
14. Schultze, M. O. and K. A. Kuiken: *J. Biol. Chem.* **137:** 727. 1941.
15. Miller, L. L.: *J. Biol. Chem.* **172:** 113. 1948.
16. Engle, F. L.: Hormones in Health and Disease. New York, Macmillan. (p. 55) 1954.

LOCAL EFFECTS OF OVARY ON UTERUS AFTER COMBINED INTRASPLENIC AUTOTRANSPLANTATION IN CASTRATED RATS

EDUARD GITSCH[1,2]

Department of Anatomy, Duke University School of Medicine, Durham, North Carolina

ABSTRACT

The main question for this investigation was whether the hypertrophic ovary which results from intrasplenic transplantation in castrated rats is really also strongly functional. A secondary question was whether an endocrine organ can have an effect on a neighboring target organ without benefit of the systemic circulation as a connecting link. Specifically, if a piece of uterus be placed in the spleen of a castrated animal together with functioning ovary, will the splenic uterus be stimulated? A total of 38 immature female rats and 7 adult females were employed. In 10 of the former at the time of castration a piece of ovary and a segment of the uterus were implanted in the spleen. Thirty days later the splenic uteri were greatly enlarged, by contrast with the nearly atrophic uteri *in situ*. The difference in weight was highly significant ($P < 0.001$). Histologic evidence of estrogenic stimulation was also apparent in the splenic uterus. On the contrary, when the ovarian graft was placed in the root of the mesentery, uterine grafts in the spleen were unaffected. In the absence of an ovarian graft, splenic grafts of uterus were again no different in weight than the uteri *in situ*.

To test the possible effect of distance separating ovary and target organ within the substance of the spleen, adult females were chosen because of their greater splenic volume. At castration the ovarian fragment was inserted in one pole of the organ and the uterine fragment in the other, to insure against a direct vascular link through an intervening scar. Thirty days later the splenic uterus remained almost as heavy as corresponding portions removed at the time of operation, while portions remaining *in situ* were significantly reduced in weight ($P < 0.05$). Therefore, it is evident that the rapidly growing ovarian tissue in the spleen of castrated rats is not only strongly active but its effect can be exerted at a considerable distance through intact splenic pulp.

IT IS well known that ovaries implanted in the spleen of castrated rats grow rapidly beyond the normal size (1, 2) showing follicles and, especially, large corpora lutea in increasing number (2). Similar results were observed in guinea pigs (3); details in which they differ from rats have been described (3, 4, 5, 6) with respect both to the ovarian grafts and to effects on the reproductive tracts. In rats enlargement of the intrasplenic ovaries

Received July 31, 1957.

[1] Lederle Fellow in Endocrinology.

[2] Present address: II. Universitäts-Frauenklinik, Wien IX, Spitalgasse 23, Österreich (Austria).

was observed in company with absence of periodic cornification of the vagina and with progressive atrophy of the vagina and uterus during the first several months (2). It has been concluded that an inactivation of estrogens produced by the intrasplenic ovary takes place in the liver. This is supported by the fact that animals with injured liver parenchyma, for instance in vitamin B deficiency (7) or after treatment with carbon tetrachloride (8, 9), lose the ability to inactivate estrogens. As a consequence of the inactivation there is loss of control of release of gonadotropins from the pituitary gland. Their increased rate of secretion brings about a rapid growth of the ovarian grafts. Histologically these appear to be hyperactive,

TABLE 1. COMPOSITION OF THE VARIOUS GROUPS

Group	Age group	Castrated or normal	Site of:		Number of rats
			Transplanted ovary	Transplanted uterine segment	
I	Immature	Castrated	Caudal pole of spleen	Caudal pole of spleen	10
II	Immature	Castrated	———	Caudal pole of spleen	8
III	Immature	Castrated	Root of mesentery	Caudal pole of spleen	6
IV	Immature	Castrated	———	———	8
C	Immature	Normal	———	———	6
V	Adult	Castrated	Cephalic pole of spleen	Caudal pole of spleen	7

yet the fact that the liver inactivates estrogens makes it impossible to recognize their effects by observation of the usual target organs. Kullander (10) demonstrated that vaginal tissue implanted in the spleen of castrated rats together with ovarian tissue shows some histological effects of the ovarian hormones which cannot be recognized in the vagina *in situ*. This approach thus seems to afford a means of estimating the extent of activity of the spleen-grafted ovary, especially if quantitative methods are used.

In the experiments described below, the uterus was chosen as target tissue because it is not only histologically a good indicator but it is also well suited by its physical characteristics for weighing procedures. As the experiments progressed, a secondary question arose: When an influence on the target tissue takes place, is this dependent on the distance between both grafts and on the character of the intervening host tissue?

MATERIALS AND METHODS

The animals used in this study were female rats, 38 immature and 7 adult, belonging to an inbred line of the Osborne-Mendel strain. The immature rats were 38–40 days old and weighed 85–90 gm. at the beginning of the experiment. The vagina was still closed except in 4 cases. The adult animals were about 180 days of age and weighed 170 to 235 gm. at the time of operation. The immature rats were assorted among four experimental groups (I–IV) and one control group (C). The adult animals formed group V. Table 1 shows the distinguishing characteristics of the several groups of experiments.

In Groups I–IV laparotomy was performed under ether anesthesia when the animals were 38–40 days old, both ovaries were removed and, except in Group IV, the cephalic

12 mm. of the left uterus was excised for transplantation. In Groups I, II and III a piece of one ovary (average weight 5 mg.) was also prepared for transplantation. The method of implanting the pieces of ovary and uterus in the spleen may be illustrated by the procedure used in Group I. The instrument for implanting the ovarian piece is a slightly curved cannula (length 7 cm., diameter 1.5 mm.) having an oblique point and fitted with a retractable stylet whose tip is bevelled to match the cannula (11). The instrument for implanting the uterine section is simply a slightly curved probe (6 cm. long, 1 mm. diam.) with a blunt point. The piece of ovary was placed just inside the oblique orifice of the cannula with the stylet slightly withdrawn. With fine scissors the fallopian tube and fatty tissue were trimmed from the segment of uterus, after which the probe was inserted a short distance into the uterine lumen. The spleen was brought into the abdominal opening, and the cannula bearing the ovarian fragment was inserted into the caudal pole. This entrance was always on the wall of the spleen that faced the stomach. The point of the cannula went 1.5–2 cm. inside and then by pushing the stylet the ovarian piece was implanted. After the cannula was removed the uterine segment was implanted by the probe through the same entrance. The spleen was gently pressed with thumb and forefinger to prevent escape of the uterine transplant as the probe was withdrawn. Because the wound in the spleen was so small, usually with no bleeding, a suture was unnecessary. In the one case in which an adhesion occurred, it was not connected with the abdominal wall, but to the mesentery root. In this case the result was no different than in the other animals.

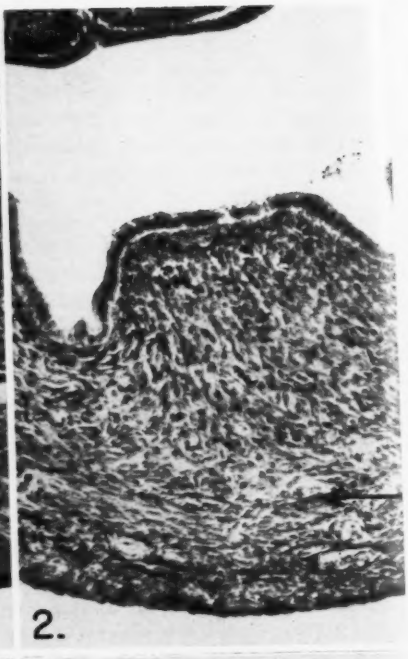
Group V, comprising the 7 adult animals, was treated in the same way as the first group except for 2 differences: (a) The ovarian piece and the uterine segment were implanted in opposite poles of the spleen using separate entrances. The ovarian piece was grafted in the cephalic pole, whereas the uterine piece was implanted in the caudal pole. Thereby one prevents the creation of possible vascular connections between the two grafts. (b) During this operation the cephalic 12 mm. of the right uterus was removed. After relaxing it with alcohol (see below) a piece 7 mm. long was weighed for later comparison with the weight of the spleen-grafted uterus.

Each animal in Groups I–V was killed with pentobarbital exactly 30 days after the operation, and each one of the control group was killed at an age corresponding with the terminal age of the rats in Groups I–IV. Ovarian and uterine grafts and corresponding pieces of the uteri *in situ* were removed to physiological saline. With the aid of binocular loupes, the uteri were cleaned for comparison of weight. In cases with distended uteri, they were first weighed and then punctured. After discharging the fluid by pressure and collecting it for examination, the tissue of the graft was weighed again. The loss represented the weight of the fluid. From the uteri a 3 mm. piece was cut off for histological examination. These and the ovarian grafts (10) were fixed in Bouin solution and stained by a modified Mallory triacid technique (12). To obtain a fair comparison of weights between the uterine graft and the uterus *in situ* the following method was devised. In order to get relaxation of the uterine musculature 95% alcohol was slowly added by medicine dropper to the physiological saline while the uteri were gently stretched, until an alcohol concentration of about 80% had been reached (approximately 45 seconds). The uteri were then dried on filter paper and cut to a uniform length of 7 mm. They were weighed individually on a torsion balance. Relative weights were expressed as milligrams per 100 gm. body weight. Differences in the average relative weight of the several groups were evaluated by means of Fisher's "t" test.

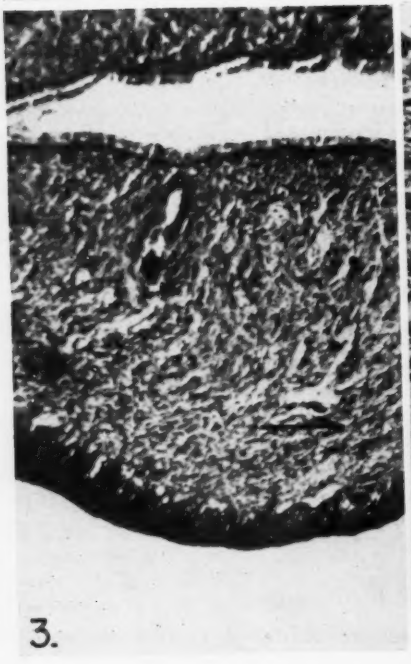
RESULTS AND DISCUSSION

In Group I, 30 days after operation there was hypertrophy of the intrasplenic uterine graft, in contrast to the nearly atrophic uterus *in situ*. In 4 cases the grafted uterus was greatly distended with a clear fluid (between

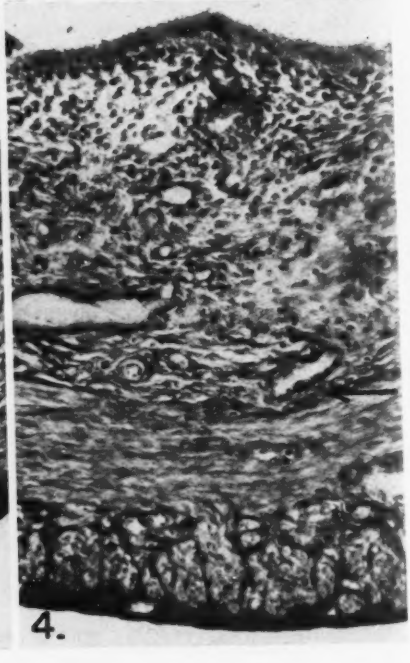
1.



2.



3.



4.

23 and 28 mg.) containing a few leucocytes. The other 6 cases appeared like normal estrous uteri and were only microscopically distended (Fig. 1). Although it is known that mechanical distention (13, 14, 15) will induce a local uterine hypertrophy without any hormonal influence, the fact that the 6 rats without grossly visible distention showed the same histological structure as the others indicates a true hormonal effect. Histologically the most impressive fact was hypertrophy of the myometrium, the combined result of increased volume of individual fibers and of an increase in fiber number. The former indicates (16, 17) an influence of estrogen.[3] Mitosis of the muscle nuclei,[3] on the other hand, is described as a progesterone effect (18, 19). The endometrial stroma was thinner than in the normal diestrus stage (Fig. 4) and similar to that in full estrus. It was edematous and the nuclei more prominent and vesicular. The thin stroma (20, 21, 22) and the edema (23) indicate an estrogen influence, whereas the nuclear characteristics indicate progesterone activity (23).[3] The epithelium was frequently destroyed by distention (Fig. 1) and, therefore, could not consistently be used as an indicator. In all usable cases it consisted of tall columnar cells with basally located nuclei. A considerable number of mitoses were visible. This also can be attributed to ovarian hormone influence.

The uteri *in situ* appeared atrophic macroscopically. Their average relative weight (av. rel. wt.) is significantly lower than that of the intrasplenic uteri (Table 2). Figure 2 is an example of the uterus *in situ* in Group I. The difference between this and the well-known atrophic uterus (Fig. 3) in castrated rats is that the endometrium is here a little thicker, and the stromal nuclei are not as pycnotic as in the endometrium of castrates (Figs. 2 and 3). Furthermore, the lumen is not as slit-shaped as in castrates. Thus, it appears that the uterus *in situ* is under a slight hormonal influence, chiefly from progesterone (10, 24, 25, 26).

The reported differences in Group I demonstrate that under direct influence of ovarian tissue a nearby graft of uterus shows consequences of both ovarian hormones. It is important to emphasize that, although the ovaries

[3] These histological interpretations are necessarily in terms of the known reactivity of uteri *in situ*. Firm conclusions must await studies of the reactivity of intrasplenic uteri in castrates treated with sex steroids.

Sections of uteri in comparable magnifications (180×). Arrow points limit inner boundary of muscularis.

FIG. 1. Intrasplenic uterus from animal also bearing intrasplenic ovary. Tall epithelium, thin endometrial stroma and heavy muscularis.

FIG. 2. Uterus *in situ* from same animal as in Figure 1. Lower epithelium, thicker endometrial stroma and thin muscularis.

FIG. 3. Uterus *in situ* from a castrated rat. Atrophic epithelium, compact endometrial stroma, thin muscularis.

FIG. 4. Uterine wall from normal cycling rat in diestrus.

TABLE 2. COMPARISON OF RELATIVE WEIGHTS OF UTERI

Group	No. of animals	Average body weight in gm. (terminal)	Uterine segment			t	P
			Identity	No.	Average rel. weight* in mg. ± S.E.M.		
I	10	174.0	SG	10	8.48 ± 0.5	5.15	<0.001
			RU	10	3.10 ± 0.06		
II	8	170.0	SG	8	2.27 ± 0.09	0.09	>0.90
			RU	8	2.28 ± 0.09		
III	6	170.0	SG	6	2.35 ± 0.05	0.02	>0.90
			RU	6	2.36 ± 0.05		
IV	8	157.0	LU	8	2.58 ± 0.07	0.03	>0.90
			RU	8	2.60 ± 0.04		
C	6	160.0	RU	6	6.99 ± 0.47	0.05	>0.90
			LU	6	7.09 ± 0.58		
I	10	174.0	RU	10	3.10 ± 0.06	0.779	>0.40
IV	8	157.0	RU	8	2.60 ± 0.04		
I	10	174.0	SG	10	8.48 ± 0.5	1.815	>0.05
C	6	160.0	RU	6	6.99 ± 0.47		<0.10
V	7	224.0	SG	7	5.81 ± 0.39	2.26	<0.05
			RU	7	2.57 ± 0.05		

* Weight of standardized 7 mm. segments of relaxed uterus in mg./100 gm. body weight.
S.E.M. = standard error of the mean.
Group I—Castrated immature female rats with ovary and uterus in spleen.
Group II—Castrated immature female rats with only uterus in spleen.
Group III—Castrated immature female rats with ovary in the mesentery and uterus in spleen.
Group IV—Castrated immature rats.
Group C—Intact immature rats.
Group V—Adult castrated female rats; ovary and uterus in opposite poles of the spleen.
SG—spleen-grafted uterine segment.
RU—tubal end of the right uterus (*in situ*).
LU—tubal end of the left uterus (*in situ*).

at 30 days after intrasplenic transplantation are almost completely made up of luteal tissue (24, 27), the intrasplenic uteri show distinct effects indicative of estrogen. In the 4 ovarian grafts of rats with distended uteri there were follicles of all sizes including a few larger than 0.4 mm. (2, 28, 29). But even in these luteinization was beginning, evidence that the number of corpora lutea was continually increasing. The older corpora lutea were somewhat compressed (24) by the newly luteinizing follicles. It is evident that the ovarian grafts were being stimulated by both FSH and LH, and the progesterone effects imply stimulation by luteotropin.

Group V was planned to find what would happen if the intrasplenic uterus and ovary are implanted in widely separated sites without possibl

connection by newly created blood vessels. A pronounced effect on the uterus was observed in all cases. The av. rel. wt. of the right uterine piece at the beginning of the experiment was 6.17 ± 0.12 mg. and that of the intrasplenic uterine transplant at the end of the experiment was 5.81 ± 0.39 mg. There was no significant difference ($t = 0.3$; $P > 0.70$). By contrast, the uterus remaining *in situ* proved to be significantly smaller than that in the spleen (Table 2). Although this difference is less extreme than in Group I, it must be realized that the animals in Group V were adult to begin with. Therefore, the two groups cannot fairly be compared. It is possible that the increased distance between the grafts in Group V may have had a limited influence. Histologically, however, the spleen-grafted uteri in the adult animals were not greatly different from those in immature rats in Group I, except that the hypertrophy of the myometrium was not as impressive in the former. For definite information on the relation between distance and *degree* of hormonal influence the reported results are insufficient. Further investigation will be needed to establish quantitative effects of distance and of the histological character of intervening tissues. The important fact for the present is that the uterus placed in the opposite pole of the spleen 20 to 25 mm. away from the ovary was nevertheless stimulated.

In Group II, castrated animals with splenic grafts of uterus alone, and in Group III, with ovary in the mesentery and uterus in the spleen, there were no significant differences in av. rel. wt. of the uteri in the spleen and of those *in situ* (Table 2). Although ovarian tissue in the mesentery undergoes the same changes as in intrasplenic ovaries, there was no comparable influence on the intrasplenic uterus as in Group I. The reason is obviously that the ovarian hormones pass directly to the liver. Furthermore, in Group IV (castrates) and in Group C (intact rats) there were no statistical differences between weights of right and left uteri (Table 2). Histologically, in Groups II and IV the uterus *in situ* was typically small and its lumen was slit-shaped (20). A few leucocytes were present in the atrophic epithelium and stroma, and the glands were collapsed and atrophic (Fig. 3). In the intrasplenic uteri of Group II there was only one difference from uteri of castrated rats, namely, a little more evident leucocytic infiltration of the subepithelial connective tissue. In Group III there was no histological difference between uteri in the spleen and *in situ*. Both were like the uterus *in situ* of Group I.

In comparing the av. rel. wt. of the uteri *in situ* of Groups I and IV there is no significant difference (Table 2). On the other hand, the av. rel. wt. of the grafted uteri of Group I is comparable with the uteri of the intact rats of Group C, the difference being of no statistical significance. This means that the intrasplenic uterus is under the direct influence of the intrasplenic ovarian tissue and able to grow and to develop, whereas the uterus *in situ* remains underdeveloped and looks atrophic. Although the atrophy is not

qualitatively as extreme as in castrated animals (10, 24), the weight difference between both uteri is not significant (Table 2).

Opening of the vagina was delayed in the animals with intrasplenic ovaries and in the simple castrates. In both Groups I and IV the vagina opened on the average 19 days after operation. This figure can be only approximate because in each group 3 animals remained closed throughout the experiment. In the rats of Group C, by contrast, the vaginas opened on the average 5 days after the operations were performed on their littermates. The age of the animals accounts for the fact that it was usually possible only to delay, but not to prevent opening of the vagina by the operation.

The results in Groups I and V give certain proof of direct hormonal influence of ovarian tissue on a target tissue grafted nearby. This confirmed statements by Kullander (10), who found that the spleen-grafted vagina reacted differently from the vagina *in situ* when ovarian tissue was grafted into the spleen in castrated rats. He presented photomicrographs which show the difference very clearly. The vaginal mucosa of the intrasplenic pieces was found degenerated into cysts which were not present in the intact vagina. Furthermore, the cysts were larger in the animals with intrasplenic ovarian tissue than in intact or castrated animals. Certain other studies have shown that ovarian hormones can exert their effects not only via the general circulation, but also by diffusion in certain circumstances. Lyons and Templeton (30) found that small amounts of estrogens applied in the vagina cause local effects without passing through the systemic circulation. Similar local effects on the uterus were reported by Grumbrecht and Loeser (31, 32). These authors also proved that estrogens have the same effect on surviving uterine tissue *in vitro*, which was demonstrated to be qualitatively the same as *in vivo*. The result in Group V of the present experiment shows that with a great distance between both grafts and with no injury to the intervening splenic pulp the hormonal influence will not be lost. The effect is not produced, therefore, by newly formed vascular links through scar tissue, as might have been supposed from the experiments of Group I.

Not only is there evidence that the intrasplenic uteri were stimulated by both estrogen and progesterone from the hypertrophic intrasplenic ovaries, but it is also noteworthy that the uterus *in situ* in Group I showed minor histological effects of progesterone. This is in accord with reports by Kullander (10) and Waidl (24). To be sure, there is evidence that the liver has some ability to inactivate progesterone (33, 34). The present observations do not deny this. They merely show that a moderate amount of progesterone is present in the systemic circulation. Perhaps a reflection of this fact was a mucification in the vaginal smears in all cases with open vagina among animals bearing splenic or mesenteric ovarian grafts. This is in agreement with other authors (10, 24).

Acknowledgments

The author wishes to express his deep appreciation and thanks to Dr. John W. Everett, not only for his careful supervision of this research, but also for his important advice which made it possible.

Furthermore he wishes to thank Mr. Terence D. George for his able preparation of histological material and Mr. Carl Bishop of the Department of Pathology for his excellent phototechnical work.

REFERENCES

1. Biskind, M. S., and G. R. Biskind: *Proc. Soc. Exper. Biol. and Med.* **55:** 176. 1944.
2. Biskind, G. R. and M. S. Biskind: *Am. J. Clin. Path.* **19:** 501. 1949.
3. Lipschutz, A.: *Nature* **157:** 551. 1946.
4. Wenner, R., and K. Hofmann: *Gynaecologia* **130:** 382. 1950.
5. Hofmann, K., and R. Wenner: *Gynaecologia* **131:** 400. 1951.
6. Stadtmüller, A.: *Arch. Gynäk.* **184:** 661. 1954.
7. Biskind, M. S. and M. C. Shelesnyak: *Endocrinology* **30:** 319. 1942
8. Talbot, N. B.: *Endocrinology* **25:** 601. 1939.
9. Pincus, G. and D. W. Martin: *Endocrinology* **27:** 838. 1940.
10. Kullander, S.: *Acta endocrinol.* **22:** 3. 1954.
11. Artner, J. and L. Velikay: Personal communication.
12. Everett, J. W.: *Endocrinology* **32:** 285. 1943.
13. Markee, J. E. and J. C. Hinsey: *Anat. Rec.* **61:** 311. 1935.
14. Markee, J. E., W. M. Wells and J. C. Hinsey: *Anat. Rec.* **64:** 221. 1936.
15. Allen, E., G. M. Smith and S. R. M. Reynolds: *Proc. Soc. Exper. Biol. and Med.* **37:** 257. 1937.
16. Allen, E.: *Cold Spring Harbor Symposia on Quant. Biol.* **5:** 104. 1937.
17. Barks, O. L. and M. D. Overholser: *Anat. Rec.* **70:** 401. 1938.
18. Crandall, W. R.: *Anat. Rec.* **70** (Suppl. 3): 18. 1938.
19. Crandall, W. R.: *Anat. Rec.* **72:** 195. 1938.
20. Allen, E. and E. A. Doisy: *Am. J. Physiol.* **69:** 577. 1924.
21. Fluhmann, C. F.: *Am. J. Obst. and Gyn.* **15:** 783. 1928.
22. Fluhmann, C. F.: *Am. J. Obst. and Gyn.* **24:** 654. 1932.
23. Hooker, C. W.: *Anat. Rec.* **93:** 333. 1945.
24. Waidl, E.: *Arch. Gynäk.* **181:** 351. 1952.
25. Kullander, S.: *Acta endocrinol.* **21:** 65. 1956.
26. Kullander, S.: *Acta endocrinol.* **21:** 204. 1956.
27. Desclin, L.: *Ann. d'endocrinol.* **15:** 1051. 1954.
28. Gitsch, E. and H. Tulzer: *Gynaecologia* **134:** 126. 1952.
29. Gitsch, E. and H. Tulzer: *Ztschr. Geburtsh. Gynäk.* **136:** 149. 1952.
30. Lyons, W. R. and H. J. Templeton: *Proc. Soc. Exper. Biol. and Med.* **33:** 587. 1936.
31. Grumbrecht, P. and A. Loeser: *Arch. Exper. Path. u. Pharmakol.* **190:** 356. 1938.
32. Grumbrecht, P. and A. Loeser: *Arch. Gynäk.* **167:** 373. 1938.
33. Dosne, Ch.: *Cancer Research* **4:** 512. 1944.
34. Engel, P.: *Endocrinology* **38:** 215. 1946.

INFLUENCE OF THE ANTICHOLINERGIC DRUG PATHILON ON THE REPRODUCTIVE CYCLE OF THE FEMALE RAT[1]

EDUARD GITSCH[2,3] AND JOHN W. EVERETT

Department of Anatomy, Duke University School of Medicine, Durham, North Carolina

ABSTRACT

A new anticholinergic drug, 3-diethylamino-1-cyclohexyl-1-phenyl-1-ethiodide (Pathilon, Lederle), was tested for its capacity to block ovulation in rats. Thirty adult females were injected with the drug (25, 50 or 75 mg./kg. body wt., s.c.) before the "critical period" on the day of proestrus. The higher doses blocked ovulation in 70% and 95% of the cases, repsectively. Five of 6 animals injected after the critical period with 75 mg./kg. ovulated normally. In 16 rats kept for later observation there was prolongation of diestrus (12–16 days) after postponed or normal ovulation; 11 were pseudopregnant, as proven by deciduoma formation.

Effects of administration during diestrus were examined in 40 rats by injecting a single dose (50 mg./kg.) on diestrus days 1, 2 or 3. There was often an effect on the immediate cycle, either prolongation of the current diestrus (21 rats, 10 pseudopregnant) or prolonged estrus with delayed ovulation (6 rats). In 10 rats prolongation of diestrus followed normal or delayed ovulation; pseudopregnancy was demonstrated in 8. Indications of FSH and LH deficiencies were seen in the histology of ovaries removed during prolonged diestrus, especially when the decidual test was negative. Corpora lutea were well developed in the pseudopregnant animals and markedly defective where the decidual reaction had failed.

SEVERAL reports from this laboratory have described experiments in which certain drugs were used successfully to prevent spontaneous ovulation in rats. From this kind of investigation it was concluded that the release of the gonadotropic hormones, especially LH, is dependent on the hypothalamus and a neurohumoral link to the pituitary gland. Adrenergic-blocking agents of the β-haloalkylamine series as well as anticholinergic substances like atropine (1, 2) are able to block the ovulation mechanism, provided that these drugs are given before the "critical period" on the day of proestrus (3). It was shown that the hypophysial activation is highly predictable in the local colony with respect to time of day, taking

Received July 31, 1957.

[1] This investigation was supported in part by a grant from the Research Council of Duke University.

[2] Lederle Fellow in Endocrinology.

[3] Present address: II. Universitäts-Frauenklinik, Wien IX., Spitalgasse 23, Österreich (Austria).

place within a restricted interval (2 to 4 P.M.) during the afternoon of proestrus (2, 3). Moreover, both Dibenamine and atropine will prevent estrogen-induced (4) and progesterone-induced ovulation in rats (5), whereas another substance prevents ovulation after injection of sheep hypophysial LH. In rabbits, the antiadrenergic agents of the Dibenamine series (6) and the anticholinergic drugs atropine and Banthine (7) are able to prevent the postcopulatory ovulation. In the hen, the blockade of ovulation, normal or prematurely induced by progesterone, has been reported after administration of Dibenamine, Dibenzyline, SKF-501 and atropine (8, 9, 10).

Older investigations with epinephrine and pilocarpine in long-period treatment (11, 12, 13) in rodents demonstrated influences of neurovegetatively effective substances on ovarian function. Swingle *et al.* (14) noted the occurrence of pseudopregnancy in rats after epinephrine injections. Nordmeyer (15) and Willig (16), in accord with Emanuel (13), reported a shortening of the cycle interval in mice after pilocarpine and apomorphine and a prolongation after atropine administration.

Besides these drugs that act on the autonomic nervous system, derivatives of barbituric acid have been especially effective on the female reproductive cycle. Westman (17) observed that female rats injected twice daily with Prominal for 3 weeks exhibited irregular vaginal cycles. In several of the animals a persistent full cornification was observed throughout the period of treatment. Several others showed a prolongation of diestrus or a complete cessation of the cycle. It is possible that Westman's persistent-estrus group corresponds with animals reported by Everett and Sawyer (3) which demonstrated that pentobarbital will prevent the ovulatory activation of the hypophysis when administered at 2 P.M. during proestrus. Persistence of graafian follicles for 2 to 3 days results when pentobarbital treatment is repeated on successive afternoons at the same critical hour. Also in agreement with Westman, Gitsch (18) observed in some animals a prolongation of the proestrus stage, and Döring and Göz (19) observed persistent vaginal cornification in a number of animals, especially if the treatment started during proestrus. Possibly related to Westman's prolonged-diestrus and anestrus groups were rats which showed a prolongation of the cycle interval while receiving daily injections of Pentothal (18) or phenobarbital (19).

From the reported investigations, especially those of Everett and Sawyer (3), it is clear that the different effects (prolongation of diestrus, proestrus or estrus) obtained with the same substance depend on the timing of administration with respect to stage of the cycle and time of day. Such had already been demonstrated (20) by the fact that whereas progesterone suppresses ovulation when its injection is begun early in diestrus, it facilitates ovulation if injected when estrogen levels are elevated. Such facilitation, in turn, depends on the time of day (5) at which progesterone is injected.

A new anticholinergic drug, 3-diethylamino-1-cyclohexyl-1-phenyl-1-

ethiodide (Pathilon, Lederle), has recently become available. This is a quaternary salt more potent than Banthine, apparently longer acting, and with a wider therapeutic margin. We were interested to learn whether this drug would have any effect on the ovulatory mechanism comparable with atropine or Banthine. Furthermore, it was of interest to find what effects it might have on the cycle when given in single injection on one or another day of diestrus.

MATERIALS AND METHODS

Mature female albino rats of the locally inbred Vanderbilt (Osborne-Mendel) strain were maintained in a room which was temperature-controlled and artificially illuminated, receiving 14 hours of light per day (3, 21). We used both 4-day and 5-day cyclic rats of a weight from 200–240 gm. A history of at least 3 uniform cycles in sequence immediately preceding the experimental cycle was required of each rat. It was previously stated (3) that the regularity of cycles in this stock is such that under these circumstances there is a probability of 93% that the next cycle will also be of this length. Ovulation in the 4-day cyclic rats occurs with regularity between 1 and 2:30 A.M. (20), 9 to 11 hours after hypophysial activation on the day of proestrus (1).

The solution of Pathilon was prepared by dissolving 5 mg. of the powder in 1 cc. physiological saline. This solution was injected subcutaneously in the lumbar region. For testing the capacity of the drug to block ovulation, 3 groups of 4-day cyclic rats, 10 rats to the group, were injected on the day of proestrus between 1:20 and 1:30 P.M. Therefore, the time of injection was approximately half an hour before the beginning of the critical period (3). The respective dosages received by the three groups were 25, 50 and 75 mg./kg. body weight. Six control rats were injected with Pathilon (75 mg./kg.) on the day of proestrus at 4:30 P.M., after the critical period had passed. On the following day, the day after the normally expected ovulation, the abdomen was opened under ether anesthesia and the reproductive tract examined. The oviducts were excised, compressed in physiological saline under a cover slip and examined under a compound microscope for tubal ova (22). The complete absence of ova in both tubes together with absence of distention of the ampullae *in situ* indicated a block of ovulation. Finding of fewer than 5 ova all together in both tubes was recorded as a partial block (1, 2). Moreover, it was noted whether the uteri were still in the hyperemic, distended proestrous condition. The ovaries were examined *in situ* under the dissecting microscope for the presence or apparent absence of unruptured follicles. The animals in doubt were killed with chloroform and the ovaries removed for histological examination. The routine staining procedure was a modified Mallory tri-acid technique (23).

The remaining animals were kept for further observation during the succeeding weeks to see what would happen in the postoperative period. As a control group, 6 other mature female rats were injected with physiological saline shortly before the critical period in proestrus. On the following day they also were laparotomized and checked in the same way as those just described. These rats also were observed for two or three weeks in the postoperative period.

In testing the effects of Pathilon injection on different days of diestrus the standard treatment was s.c. injection of 50 mg./kg. body weight at about 1:30 P.M. Four groups (10 animals in each group) were employed. The rats in 3 groups had histories of 5-day cycles, and those in the other group had histories of 4-day cycles. Injections were given to the 5-day cycling rats on the first, second or third day of diestrus. The 4-day cycling rats were injected on the second day of diestrus.

All animals were controlled daily by vaginal smear. When a prolongation of diestrus

curred after injection, the rats were laparotomized on the fifth diestrus day and the terus was traumatized. Four days later it was examined at laparotomy for deciduomata. very second animal was subjected at that time to unilateral ovariectomy, the one vary being preserved for histological examination. After the end of pseudopregnancy or milar prolongation of the cyclic interval the vaginal smearing was continued through cycles.

RESULTS AND DISCUSSION

Pathilon was well tolerated without evident side effects, except possibly in the third (75 mg./kg.) group injected during proestrus, in which 4 ani-

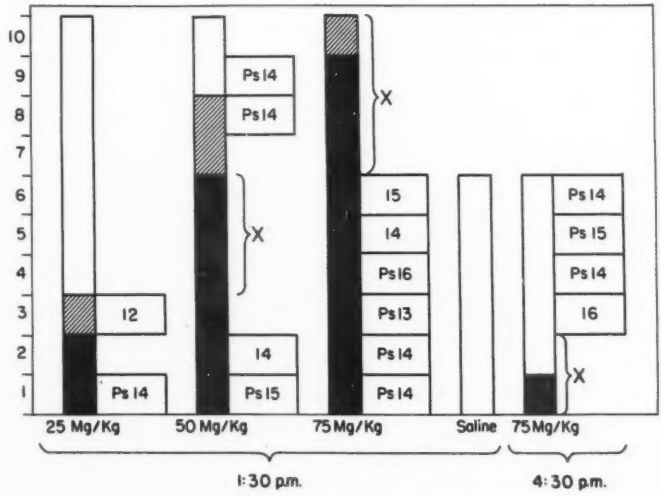


FIG. 1. Comparative results in 5 groups of animals injected with different amounts of Pathilon or with physiological saline. In the vertical columns are shown the results with respect to ovulation: solid black—complete blockade; cross hatching—partial blockade; white—complete ovulation. The times of injection are indicated below the brackets. On the left vertical scale is shown the number of animals. The flags on the right side of each column represent prolongation of diestrus following the current estrus. *Ps* indicates prolongation with decidual reaction, i.e. pseudopregnancy. If no flag is shown the following cycles were normal. Rats which were killed or which died "spontaneously" are inicatedd by a cross.

mals were found to have a local wound reaction after repeating the injection, and 2 others died of pneumonia.

Series I. The effects of injection during proestrus are graphically presented in Figure 1. In the first (25 mg.) group only 3 of the 10 animals were affected by the drug in any evident way. In two of these ovulation was blocked and postponed one day. One of them had a prolongation of proestrus, and vaginal estrus occurred for the first time on the second day after injection; in the other case estrus occurred as expected on the first

day after injection but remained until the second day, at which time fresh ovulation was demonstrated. In this case a pseudopregnancy followed. In the 3rd rat a partial block was found on the day after injection. The following diestrus was prolonged to 12 days, but no decidual reaction was evoked.

In the second (50 mg.) group, 9 of the 10 rats were affected in some way. Ovulation was blocked in 6 animals. In 2 of these proestrus was prolonged one day; in one case, 2 days with consequent postponement of ovulation for 2 days; and in the other 3 rats estrus occurred on the day after injection and remained one additional day. Among these six animals, 2 had prolongation of the following diestrus to 14 and 15 days, one with and one without decidual reaction; one rat had normal cycles after the blockade; and the other three were killed for examination. In addition to the six, 2 other animals showed a partial block, one of them subsequently showing a pseudopregnancy, the other returning promptly to normal cycles. Finally, the 2 remaining rats ovulated normally. One of them afterward experienced a pseudopregnancy.

In the third group, ovulation was completely blocked in 9 of the 10 animals and partially blocked in the other after 75 mg./kg. Pathilon was administered on the day of proestrus. Two rats, after blocked (i.e. postponed) ovulation, exhibited a prolongation of diestrus for 14 and 16 days, one having a decidual reaction. Two animals were blocked 2 days and during this period they showed a persistent estrus; one of them continued with a pseudopregnancy, the second simply with a prolongation of diestrus. In 5 rats injection was repeated on the second day. One rat, after having been fully blocked by the first injection, was only partially blocked by the second one, having 4 fresh tubal ova when observed at autopsy on the following day. The other four of the doubly injected rats remained blocked: two for 2 days, with prolongation of proestrus; two for 3 and 4 days, respectively, with prolongation of estrus. Two of the 4 rats died of pneumonia on the sixth and seventh days. The two that survived became pseudopregnant after the postponed ovulation.

In the group of 6 control rats injected with Pathilon (75 mg./kg.) at 4:30 P.M. on the day of proestrus, 5 animals ovulated during the night and one animal was blocked. This result is in accord with the expectation for this hour of injection (24). Four of these rats were studied subsequently; three became pseudopregnant and one had a prolongation of diestrus (16 days) without decidual reaction to trauma.

In the control group of 6 animals injected with physiological saline preceding the critical period, we observed normal ovulation; subsequent cycles were also normal. It should be noted that pseudopregnancy was absent in spite of laparotomy on the day after injection.

It is apparent that after increasing the dosage of Pathilon the proportionate number of blocked ovulations also increased. In the 25 mg. group

25% were blocked.[4] The figure increased to 70% and 95%, respectively, in the 50 mg. and 75 mg. groups. If we compare these percentages with the expected frequency of spontaneous delay of ovulation in this stock, ca. 5% (3), we obtain the following results, using the contingency tables of Mainland and Murray (25): in the 25 mg. group the observed frequency of blockade is not significant, that in the 50 mg. group is significant at the 5% level and that in the 75 mg. group at the 1% level. In fact, in even the 25 mg. group the one case of partial blockade clearly indicates a drug effect.

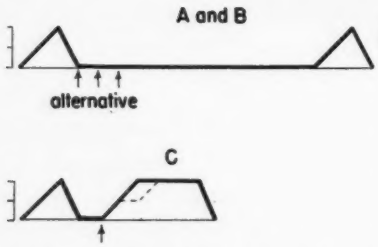
Thus, it has been demonstrated that Pathilon is able to block ovulation similarly to Banthine and atropine. In addition, it was observed that after the blockade or even after normal ovulation following Pathilon, in a variable percentage of the cases the following expected cycle was replaced by a prolonged diestrus lasting between 12 and 16 days. Whereas in the 25 mg. group only a few rats reacted in this way, in the 75 mg. group there were pseudopregnancies in all of the postoperatively observed cases.

Altogether, in the various phases of Series I, there were 16 animals with prolongation of diestrus, of which 11 were pseudopregnant. Ovaries of 3 of the latter were available for histological study. Removed on the ninth day, they disclosed no difference from what one would expect to find in normal pseudopregnancy. On the other hand, the ovaries from 3 rats that failed to respond with a decidual reaction were deficient in various respects. In one the luteal tissue was restricted to a peripheral shell about a massive core of connective tissue. In the 2 others, although the corpora lutea were compactly developed, the luteal cells were of widely varying size, relatively few being as large as they normally should be. In all 3 defective ovaries there was evidence of inadequate FSH and LH stimulation. The follicles were not over 0.4 mm. diameter and the prominent ones were usually in various stages of atresia. The interstitial tissue of 2 of the 3 ovaries contained abundant wheel nuclei, indicative of LH deficiency (26).

Series II. We turn now to the second question: how would the animals differ in their reactions to a standard single dose of Pathilon (50 mg./kg.) injected on different days of diestrus? In considering the results one may differentiate two sets of characteristic effects: those expressed within the cycle during which injection was given (Fig. 2), and those expressed after a normal or briefly prolonged cycle (Fig. 3). The character of the effects varied with respect to the timing of the injection. A detailed account follows.

Injection on the first day of diestrus in 5-day cyclic rats always resulted in a prolongation of the current diestrus, which lasted between 13 and 16 days; 6 rats were pseudopregnant whereas 4 animals showed a simple pro-

[4] In calculating such percentage, a case of partial blockade is arbitrarily given a value of 5% and one of total blockade receives a value of 10%, one animal equalling 10% of the total.



Day of Injection	Kind of Cycle	A	B	C	O
1	5-day	6	4	0	0
2	5-day	1	4	1	4
2	4-day	1	0	4	5
3	5-day	2	3	1	4

FIG. 2. Schema and tabulation showing various effects of Pathilon injection during diestrus on the current cycle. A: pseudopregnancy. B: prolongation of diestrus without decidual reaction. C: prolongation of proestrus (broken line) and estrus. O: no reaction on the current cycle. Various days of injection represented by arrows. One vertical unit indicates proestrus; two units, full cornification.

longation of diestrus without decidual reaction. In one which was followed longer a second prolongation was observed to last 15 days, without decidual reaction.

After injection on the second diestrus day in 5-day cyclic rats, one reacted with pseudopregnancy twice in succession. In 4 rats there continued a simple prolongation of diestrus. One animal had a 16-day prolongation of diestrus after 2 intervening periods of cyclic estrus. One other had a postponement of ovulation for 2 days, accompanied by prolongation of vaginal estrus to 3 days. The same animal afterward experienced pseudopregnancy. Three rats were entirely unaffected.

In the group of 4-day cyclic rats the following results were found after injection on the second day of diestrus: 5 rats showed an effect within the current cycle and 5 did not. Of the latter, two rats had a normal cycle after the expected ovulation and then became pseudopregnant, 1 animal reacted more immediately with a pseudopregnancy after normal estrus and ovulation, and 2 rats seemed entirely unaffected by the drug. Of the 5 that reacted within the current cycle, 1 experienced a prolongation of diestrus

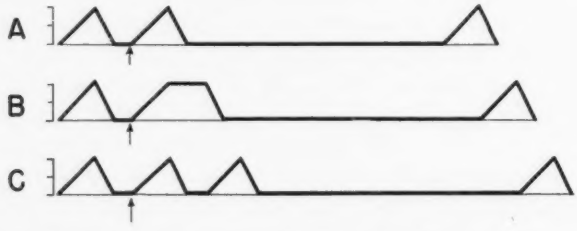


FIG. 3. Three characteristic sequences involving prolonged diestrus subsequent to ovulation. A: pseudopregnancy after normal ovulation, 3 rats. B: prolonged diestrus following delayed ovulation, 3 or 4 rats pseudopregnant. C: prolonged diestrus following two cycles, 2 of 3 rats pseudopregnant.

which was proven to be a pseudopregnancy, and 4 had a prolongation of estrus, indicative of blockade of ovulation. After their vaginal estrus appeared at the expected time on the 4th day of the cycle, estrus continued for 2 additional days in 2 rats and for 3 additional days in 2 others. In their subsequent histories, one of these four rats had a similarly prolonged estrus in the cycle immediately following, one had a simple prolongation of the following diestrus, and two became pseudopregnant (one twice in succession).

Injection on the third day of diestrus in 5-day cyclic rats gave these results: Three cases subsequently had a simple prolongation of diestrus and two others became pseudopregnant. In one additional rat ovulation was postponed for 2 days with vaginal proestrus prolonged to 2 days and estrus to 2 days. Two rats became pseudopregnant after ovulation at the expected time and, finally, two animals had no apparent reaction whatever.

Figure 2 schematically summarizes and tabulates the various effects expressed within the current cycle. The first kind of reaction is a prolongation of diestrus, either as a frank pseudopregnancy (A) or as a simple prolongation without decidual reaction (B). Although mucification of the vaginal smear was observed in either circumstance, the significance of this is questionable in group B, inasmuch as there was no histological evaluation of the vaginal mucosa at this time. A low level of estrogen may in itself cause mucification of the smear, a fact that is well recognized. Diminished estrogen output may be postulated from the histological appearance of the ovaries. They gave the same indications of FSH and LH deficiency that were noted in ovaries of similar animals in Series I: decreased size and number of growing follicles, and "wheel" structure in the nuclei of the interstitial cells.

The second kind of reaction expressed in the current cycle (Fig. 2, C) is the postponement of ovulation, accompanied by prolonged vaginal estrus. This was observed in 5 of the 20 rats that were injected on the last day of diestrus. The effect corresponds to the blockade observed in higher frequency in Series I, after injection during proestrus. A protracted blocking action of the drug was noted in some of those cases, extending beyond the first 24 hours after a single injection. Even more extremely, in some of these in Series II, blocking action continued for even three or four days—note especially the 5-day cyclic rat in which ovulation was delayed two days, after injection was made on the second day of a 3-day diestrus. Other evidence of protracted activity of Pathilon is noted in laboratory reports supplied by the manufacturer which state that it inhibits secretion of gastric HCl three times as long as Banthine. However, this is a matter of hours, not days.

Effects appearing later than the current cycle (Fig. 3) were expressed according to 3 different patterns. In all cases the overt effect was a prolonga-

tion of diestrus, and in 8 of the 10 cases this proved to be a pseudopregnancy, the decidual reaction in all eight being uniformly good. However, because of the small number of animals (three) following the third pattern, in which the long interval came after two intervening estrus periods, it is uncertain that this is truly a drug effect.

From the above observation it is apparent that the effects of Pathilon on pituitary function are multiple. Like atropine and Banthine, it can block LH release and thus prevent ovulation. But further than that, it can bring about pseudopregnancy, either in the cycle during which it is administered or in the one that follows. A recent report by Barraclough (27) demonstrates that, somewhat similarly, chlorpromazine and reserpine are able not only to block ovulation (28, 29), but also to evoke pseudopregnancy, especially when injected on the first day of diestrus. Krais, Lehr and Riess (30) had earlier found that chlorpromazine (Megaphen) delays or prevents the occurrence of the expected estrus in rats.

Perhaps one factor in the mechanism whereby Pathilon induces pseudopregnancy is its tendency to suppress the day-to-day secretion of gonadotropin (FSH-LH). Although this was most extreme in the cases of prolonged diestrus without corpus luteum function, even in the true pseudopregnancies there was sometimes a moderate tendency to wheel structure in the nuclei of interstitial cells of the ovary. There are several reasons to consider the possibility that factors which depress FSH-LH secretion will at the same time favor the secretion of luteotropin (LTH). Greep and Jones (31) noting the well-recognized fact that the administration of appropriate amounts of estrogen will cause pseudopregnancy, postulated that it does so indirectly by suppressing FSH and LH. Everett (32, 33) demonstrated that pseudopregnancy is regularly produced simply by autotransplanting the anterior hypophysis to the kidney capsule, a circumstance that causes atrophy of all parts of the ovary except the corpora lutea. Rothchild (34) finds that during normal pseudopregnancy unilateral ovariectomy is not followed by hypertrophy of the remaining ovary as would be the case in cycling animals. On the contrary, a hypothesis that FSH-LH depression is the primary factor initiating pseudopregnancy is difficult to reconcile with the phenomenon of delayed pseudopregnancy first described by Greep and Hisaw (35). Electrical stimulation of the cervix uteri during diestrus will often result in pseudopregnancy after the next expected ovulation a few days later. Essentially the same result occurs after copulation during a cycle in which ovulation is blocked by pentobarbital (36). The beginning of the pseudopregnancy awaits the return of cyclic estrus; the corpora lutea forming at this later time then become functional. Surely, the follicle maturation and the ovulation these several days after the copulatory stimulus must mean that FSH and LH remain normal meanwhile. It is interesting to note that many of our rats that were

injected with Pathilon during late diestrus experienced pseudopregnancy only after the completion of a new ovulation. The parallel with the delayed effect of cervical stimulation etc. is obvious. Explanation is equally elusive in either case.

REFERENCES

1. Everett, J. W., C. H. Sawyer and J. E. Markee: *Endocrinology* **44:** 234. 1949.
2. Everett, J. W. and C. H. Sawyer: *Endocrinology* **52:** 83. 1953.
3. Everett, J. W. and C. H. Sawyer: *Endocrinology* **47:** 198. 1950.
4. Sawyer, C. H., J. W. Everett and J. E. Markee: *Endocrinology* **44:** 218. 1949.
5. Everett, J. W. and C. H. Sawyer: *Endocrinology* **45:** 581. 1949.
6. Sawyer, C. H., J. E. Markee and W. H. Hollinshead: *Endocrinology* **41:** 395. 1947.
7. Sawyer, C. H., J. E. Markee and J. W. Everett: *Am. J. Physiol.* **166:** 223. 1951b.
8. Zarrow, M. X. and J. W. Bastian: *Proc. Soc. Exper. Biol. and Med.* **84:** 457. 1953.
9. van Tienhoven A., A. V. Nalbandov and H. W. Norton: *Endocrinology* **54:** 605. 1954.
10. van Tienhoven, A.: *Endocrinology* **56:** 667. 1955.
11. Kraul, L.: *Arch. Gynäk.* **131:** 600. 1927.
12. Hirsch-Hoffmann: *Arch. Gynäk.* **156:** 42. 1934.
13. Emanuel, R.: *Ztschr. Geburtsh. Gynäk.* **124:** 44. 1942.
14. Swingle, W. W., P. Seay, J. Perlmutt, E. J. Collins, G. Barlow, Jr., and E. F. Fedor: *Am. J. Physiol.* **167:** 586. 1951.
15. Nordmeyer, K.: *Zbl. Gynäk.* **74:** 2. 1952.
16. Willig, H.: *Zbl. Gynäk.* **74:** 17. 1952.
17. Westman, A.: *Acta med. Scand.* **128** (Suppl. 196): 111. 1947.
18. Gitsch, E.: *Zbl. Gynäk.* **72:** 945. 1950.
19. Döring, G. K., and E. M. Göz: *Arch. Gynäk.* **181:** 185. 1952.
20. Everett, J. W.: *Endocrinology* **43:** 389. 1948.
21. Everett, J. W.: *J. Clin. Endocrinol. & Metab.* **12:** 923. 1952b.
22. Everett, J. W.: *Endocrinology* **41:** 364. 1947.
23. Everett, J. W.: *Endocrinology* **32:** 285. 1943.
24. Everett, J. W.: *Endocrinology* **59:** 580. 1956.
25. Mainland, D. and I. M. Murray: *Science* **116:** 591. 1952.
26. Selye, H., J. B. Collip and D. L. Thomson: *Endocrinology* **17:** 494. 1933.
27. Barraclough, C. A.: *Anat. Rec.* **127:** 262. 1957.
28. Barraclough, C. A.: *Anat. Rec.* **124:** 255. 1956.
29. Barraclough, C. A.: *Fed. Proc.* **14:** 9. 1955.
30. Krais, W., H. Lehr and W. Riess: *Arch. Gynäk.* **185:** 40. 1954.
31. Greep, R. O. and I. C. Jones: *Recent Progr. Hormone Res.* **5:** 197. 1950.
32. Everett, J. W.: *Endocrinology* **54:** 685. 1954.
33. Everett, J. W.: *Endocrinology* **58:** 786. 1956.
34. Rothchild, J.: unpublished.
35. Greep, R. O. and F. L. Hisaw: *Proc. Soc. Exper. Biol. & Med.*: **39:** 359. 1938.
36. Everett, J. W.: *Ciba Foundation Colloquia Endocrinol.* **4:** 167. 1952.

ESTROGEN CONTROL OF THE PHOSPHOLIPIDS OF THE UTERUS[1]

HERBERT ELFTMAN

Department of Anatomy, College of Physicians and Surgeons, Columbia University, New York, N. Y.

ABSTRACT

The influence of estradiol benzoate and of progesterone on the distribution of phospholipid in the endometrium of the castrate rat has been studied in tissue sections prepared by the controlled chromation method for phospholipids and the direct silver method for the Golgi apparatus. Estrogen decreases the basal phospholipid and causes a rapid growth of the Golgi apparatus, effects which are not paralleled by progesterone. The response of both the general phospholipid and the Golgi apparatus to graded doses of estradiol benzoate has been investigated.

INTRODUCTION

RECENT improvements in histochemical techniques for phospholipids have increased their accuracy and have, at the same time, facilitated their routine use by permitting the embedding of the tissue in paraffin. With the aid of these advances it has been possible to gain further information concerning the phospholipids of the uterus. Earlier investigations, summarized by Alden (1), have pointed out basic aspects of the problem. The purpose of the experiments reported in this paper has been to map the distribution of phospholipid in the uterus of the rat as it responds to the administration of estrogen, with some preliminary results of experiments with progesterone.

Exploratory experiments demonstrated that one of the sites of marked phospholipid change in the uterine epithelium was the Golgi apparatus. The availability of a rapid method for visualizing the Golgi apparatus allowed us to study its variations in conjunction with those of other phospholipid components.

MATERIALS AND METHODS

Rats of the Long-Evans strain, bred in the local colony, were used in all of the experiments. Females ranging in age from 5 to 8 months were ovariectomized from 18 to 27 days before hormone administration. The division of the animals into 9 groups was followed by the injection of steroid hormones and collection of tissue according to the schedule shown in Table 1. All of the hormones were dissolved in sesame oil and were given subcutaneously.

Received August 8, 1957.

[1] Aided by a grant from the National Science Foundation.

The animals in groups 1–4 constituted the first set of experiments, designed to differentiate the effect of estradiol benzoate and progesterone on phospholipid. The tissues from these groups were collected 24 hours after the last injection. When groups 5–9 were experimented with, 48 hours was allowed for the action of the last injection before autopsy since experiments not forming part of this paper had indicated that this time period provided maximum effect of the hormone on the features we were observing. It was our intention to employ dosages approaching a physiological range and to allow them to act for a period of time not too far displaced from the normal.

The tissues were collected during terminal chloroform anesthesia. Phospholipids were studied by means of controlled chromation with dichromate-sublimate fixation as

TABLE 1. DOSAGE OF HORMONES INJECTED INTO OVARIECTOMIZED RATS AND TIME ALLOWED FOR THEIR ACTION BEFORE AUTOPSY

Group	No. of animals	Treatment		Hours between last dose and autopsy
		Dose/day	No. of days	
1	7	Untreated		
2	3	2 μg. estradiol benzoate	3	24
3	3	1 mg. progesterone	3	24
4	3	2 μg. estradiol benzoate +1 mg. progesterone	3	24
5	6	0.25 μg. estradiol benzoate	2	48
6	5	0.50 μg. estradiol benzoate	2	48
7	4	1.0 μg. estradiol benzoate	2	48
8	5	2.0 μg. estradiol benzoate	2	48
9	4	4.0 μg. estradiol benzoate	2	48

described by Elftman (2, 3). The samples of tissue were placed in fixative freshly prepared by dissolving potassium dichromate to the extent of 2.5% in a stock 5% solution of mercuric chloride, the pH being adjusted to 2.5 with HCl, the measurement being made with a Beckman pH meter. After four days in the fixative at room temperature the tissues were embedded in paraffin, passing through two 10 minute changes of xylol in the process. Staining with Sudan black B in ethylene glycol (4) followed.

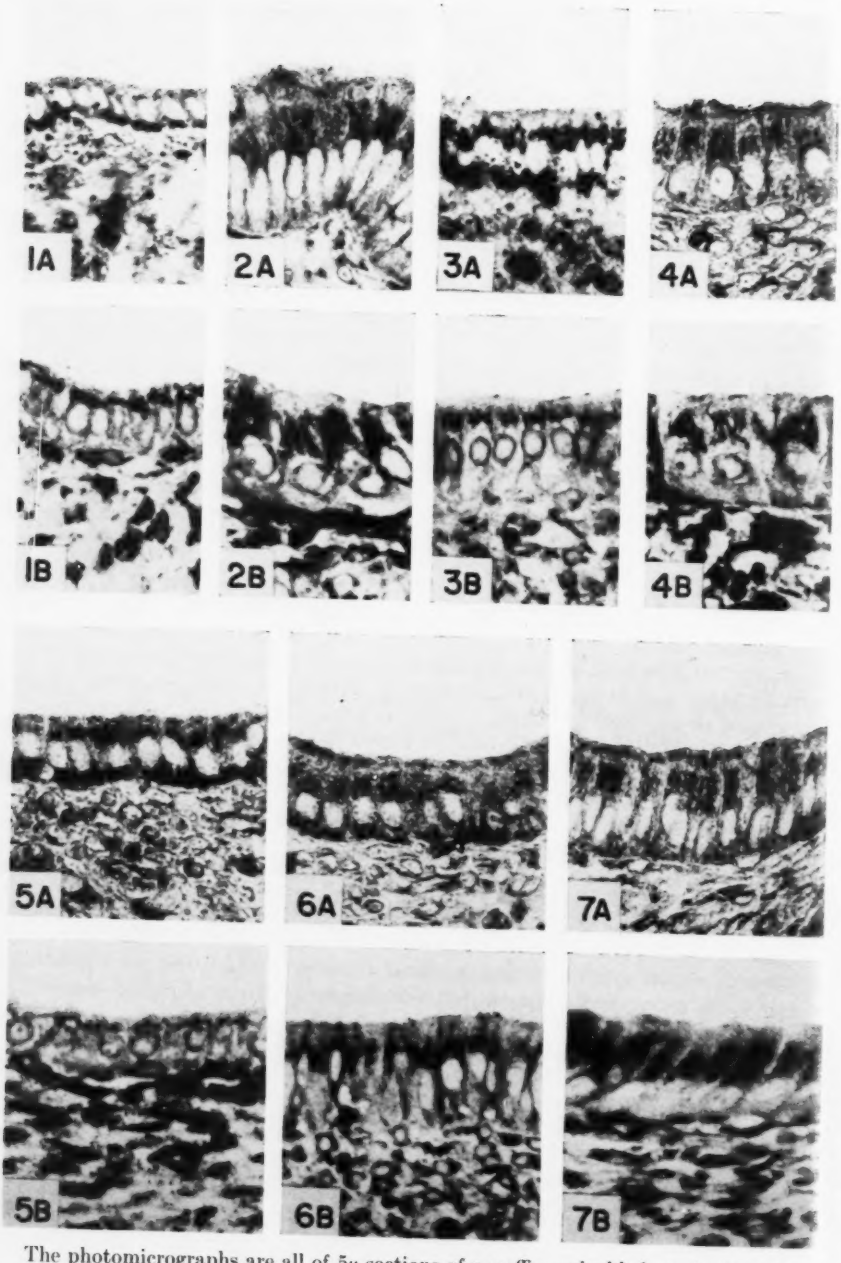
Duplicate samples of tissue were taken for study of the Golgi apparatus. The method employed was Elftman's (5) direct silver. The tissue was immersed directly in 2% silver nitrate in 15% formalin and kept in this solution for three hours at room temperature. The silver solution was then decanted and replaced, without washing, by 2% hydroquinone in 15% formalin. Transfer to 15% formalin three hours later allowed overnight completion of general fixation. Paraffin embedded sections were counterstained with toluidine blue. For greater permanence some slides were gold-toned, the substitution of gold for silver conferring the greater resistance to oxidation which is a property of the more noble metal.

For preservation of mitochondrial detail beyond that which is apparent after controlled chromation, some tissue was put through osmichrome fixation (6) and the mitochondria stained with anilin acid fuchsin or with iron hematoxylin.

OBSERVATIONS

Uterus of the castrate

The uterine epithelium of an animal spayed four weeks earlier (Fig. 1A) shows two regions of phospholipid accumulation. The basal phospholipid stains more intensely with Sudan black B, suggesting that it is more purely lipid in character. In the apical region two phospholipid components can



The photomicrographs are all of 5μ sections of paraffin embedded uteri of ovariectomized rats, each one prepared by two methods: A) controlled chromation with Sudan black staining of the phospholipid; and B) Golgi apparatus visualization by the direct silver method. $\times$640.

be more closely identified. The convoluted mass adjacent to the nucleus is seen in Figure 1B to have given the typical Golgi apparatus reaction to silver. Closer to the lumen are particles derived from mitochondria, as evidenced by their reaction to silver and their staining by hematoxylin and by anilin acid fuchsin after osmichrome fixation. Since it is well known that both the Golgi apparatus and the mitochondria contain protein in addition to phospholipid, it is understandable that the Sudan black B stain should be less intense in the apical region.

Effect of estrogen

Three daily injections of 2 μg. of estradiol benzoate resulted in the phospholipid distribution of Figure 2, 24 hours after the last injection. The minimal Golgi apparatus of the castrate has developed into a luxuriant form with the phospholipid expanded as an interfacial film. This makes it difficult to estimate the amount of phospholipid by inspection; when quantitative results are needed they may be obtained by the methods suggested in (7).

The effect of estrogen in decreasing basal phospholipid is almost as dramatic as its influence on apical agumentation. The possibility of transfer of phospholipid from base to apex is apparent but, if it does take place, it must involve not only geographical translocation but also chemical incorporation into the more complex apical structures.

Effect of progesterone

The administration of 1 mg. doses of progesterone according to the schedule used for estrogen influences the Golgi apparatus (Fig. 3) very little but there is an intimation that the amount of basal phospholipid may actually have increased. Since there is a regional variation in basal lipid, the antimesometrial and mesometrial domains being more richly supplied, a precise determination of the quantitative effect of progesterone awaits more extended experiments. The appearance of a clear area between

---

FIG. 1. Castrate control. In 1A there is a concentration of phospholipid in the basal region of the epithelium and a lesser amount in the apical region. The apical phospholipid is partially mitochondrial although that which is near the nucleus belongs to the Golgi apparatus. The latter is clearly visible in the direct silver preparation, 1B.

FIG. 2. After 3 daily injections of 2 μg. of estradiol benzoate. The Golgi apparatus has increased tremendously in size and the basal lipid has disappeared.

FIG. 3. After 3 daily injections of 1 mg. of progesterone. There has been no decrease in basal phospholipid nor increase in size of the Golgi apparatus.

FIG. 4. After concurrent administration of the treatments described for Figures 2 and 3. The effect is similar to that produced by estrogen alone but is more moderate.

FIGS. 5, 6 and 7 represent the effects of two daily doses of 0.25 μg., 1.0 μg. and 4.0 μg. of estradiol benzoate respectively with 48 hours elapsing before autopsy. Note the decrease in the basal phospholipid and the increase in size of the Golgi apparatus as the dose of estrogen is increased.

the Golgi apparatus and the nucleus in Figure 3B is not unusual in progesterone treated animals. Whether it is associated with non-Golgi apical lipid remains to be seen.

Effect of concurrent estrogen and progesterone

Concurrent administration of 2 μg. of estradiol benzoate and 1 mg. of progesterone for three successive days (Fig. 4) produced results reminiscent of the effects of estrogen alone but reduced in magnitude by the presence of progesterone.

Effect of graded doses of estrogen

A preliminary attempt to determine quantitatively the effect of estrogen on endometrial phospholipids by using dosages and times not too far removed from those natural to the rat is represented by groups 5–9 of Table 1. Tissues from groups 5, 7 and 9 are shown in Figures 5–7, each figure thus representing a four-fold increase in estrogen dosage.

The smallest dosage which we employed had very little influence on basal or apical phospholipid (Fig. 5). Doubling the dose to 0.50 μg. per injection produced some evidence of activity and further increase to 1.0 μg. caused the marked changes shown in Figure 6. The increase in apical lipid, both Golgi and mitochondrial, is carried still further by the 4.0 μg. dosage (Fig. 7). Basal lipid is still present in Figure 6A but in Figure 7A it has almost completely disappeared.

DISCUSSION

Uterine lipids of the rat and the mouse have been studied by histological methods of varying degrees of applicability by many investigators (1, 8–12). With the rapid development of histochemistry in recent years it has become possible to demand greater precision than was possible by the methods available to the early workers. The ease with which phospholipids can be studied by controlled chromation and the Golgi apparatus by the direct silver method permits observation of these features to be included in routine experimentation.

The decrease in basal lipid which can be produced by estrogen has been discussed by Gilbert (13) and by Alden (1) but the accompanying increase in size of the Golgi apparatus has escaped observation. The increase in apical phospholipid which is implied by the growth of the Golgi appratus, since it has an appreciable phospholipid component, takes place in fair part while the basal lipid is decreasing. It is possible that basal phospholipid may migrate apically and become incorporated in the Golgi complex but a more immediate metabolic use is likely.

The present paper is in a measure a sequel to the discovery by Atkinson and Elftman (14) that estrogen mobilizes alkaline phosphatase in the uterus of the mouse. The shift in phospholipids of the uterus which is induced by

estrogen and the luxuriant increase in the Golgi apparatus which it provokes are also links in the mechanism by means of which estrogen performs its physiological function.

Acknowledgment

This investigation owes much to the technical skill of Mary Clarke Miksic.

REFERENCES

1. Alden, R. H.: *Anat. Rec.* **97**: 1. 1947.
2. Elftman, H.: *J. Histochem. & Cytochem.* **2**: 1. 1954.
3. Elftman, H.: *Stain. Tech.* **32**: 29. 1957.
4. Chiffelle, T. L. and F. A. Putt: *Stain. Tech.* **26**: 51. 1951.
5. Elftman, H.: *Stain. Tech.* **27**: 47. 1952.
6. Elftman, H.: *Quart. J. Micr. Sci.* **98**: 15. 1957.
7. Elftman, H.: *Anat. Rec.* **118**: 147. 1954.
8. Besse, P. M. and D. Christides: *Arch. Med. Exper. et Anat. Path.* **26**: 573. 1915.
9. Bourg, R.: *C. r. Soc. Biol.* **102**: 592. 1929.
10. Reese, J. D.: *Anat. Rec.* **48**: 55. 1931.
11. Bloch, S.: *J. Endocrin.* **1**: 399. 1939.
12. Davis, J. S. and R. H. Alden: *Anat. Rec.* **127**: 283. 1957.
13. Gilbert, C.: *Endocrinology* **30**: 773. 1942.
14. Atkinson, W. B. and H. Elftman: *Endocrinology* **40**: 30. 1947.

THE METABOLISM OF I^{131}-LABELLED THYROID HORMONES IN THE HYPOPHYSIS AND BRAIN OF THE RABBIT[1]

DONALD H. FORD AND JACK GROSS

Department of Anatomy, College of Medicine, State University of New York, Brooklyn, New York

ABSTRACT

The distribution and metabolism of thyroid hormones in the brains and the hypophyses of normal rabbits was determined after intravenous administration of I^{131}-labelled triiodothyronine or thyroxine.

Triiodothyronine concentrated in the gray matter of the brain, principally in the region of the paraventricular nucleus and the median eminence, and, more heavily, in the hypophyseal lobes. The pattern of distribution of thyroxine was similar, but concentration was much less intense. Very little activity was detected in the adenohypophysis, although the existence of an extravascular thyroxine space there was determined.

Triiodothyronine rapidly left the plasma, but uptake in the tissues was slower, reaching a peak after about two hours. This pattern of concentration in time may have been dependent on the formation of a previously postulated plasma-triiodothyronine complex as a precursor of the tissue triiodothyronine. It resulted in high concentration gradients between tissues and plasma. Levels as high as 75:1 for the neurohypophysis and 17:1 for the adenohypophysis were reached; the highest for the cerebrum and diencephalon were 1.6:1 and 1.5:1, respectively.

Although thyroxine reached peak concentration in the tissues about as rapidly as triiodothyronine, its level remained more constant, a slower rate of metabolic degradation being indicated. With equal doses, about three times as much triiodothyronine as thyroxine was taken up by the tissues. This ratio may partly explain the differences in biological activity of the two substances. The uptake of each hormone was roughly proportional to the dose injected (0.5 and 4.0 μg./kg. for thyroxine; 0.5, 4.0 and 12.0 μg./kg. for triiodothyronine).

Chromatographic analysis showed the presence of triiodothyronine in the brains and the hypophyses of animals receiving triiodothyronine. The presence of iodide in the hypophyses of such animals suggested deiodination of triiodothyronine in hypophyseal tissue. No other known metabolites of triiodothyronine were detected. In thyroxine-treated animals, thyroxine was present in the brain and both triiodothyronine and thyroxine were detected in the hypophysis.

Received August 15, 1957.

[1] Supported in part by Grant No. A-370 from the National Institute for Arthritis and Metabolic Diseases of the National Institutes of Health, United States Public Health Service.

A preliminary report of this work was given before the Endocrine Society at Atlantic City in June 1955.

IN A previous study in our laboratories on the distribution of I^{131}-labelled triiodothyronine (T_3) in mice, it was noted that the hypophysis was one of the sites of heaviest concentration of radioactivity (1). In order to define this concentration more accurately, the present study was initiated. Owing to the important physiological relationship between thyroid activity and brain function, it was decided that the study should include various parts of the brain, as well as the different parts of the hypophysis, and that the distribution of thyroxine should be investigated, as well as that of triiodothyronine. The rabbit was selected as an appropriate animal for the experiments.

After intravenous injection of relatively small amounts of either hormone labelled with I^{131}, concentration of radioactivity of varying degrees of intensity was detectable in various areas of the brain and the pituitary. Some of the neural areas involved may be related to the functional control of the thyroid gland.

MATERIALS AND METHODS

The male albino rabbits, weighing between 2 and 3 kg. were obtained from a local animal supply house on the day the experiments were begun.

The radioactive triiodothyronine and thyroxine were obtained in a 50% propylene glycol solution from Abbott Laboratories, at Oak Ridge, Tennessee. Saline dilutions suitable for injection were prepared in siliconized glassware, so as to reduce losses by adsorption on the walls of the containers. The diluted triiodothyronine was analyzed chromatographically for purity and was found to contain 82% or more of the hormone. The diluted thyroxine contained only 71% of the hormone. Suitable standards of the material were prepared by dilution with 0.1N NaOH.

In a typical experiment the procedure was as follows. The animals were anesthetized by intraperitoneal injection of pentobarbital, dial urethane, or 12% ethanol. Anesthesia was maintained with ether. The external jugular veins were exposed, one vein being used for the injection of labelled hormone (0.5 μg./kg.),[2] the other for withdrawal of heparainized blood samples of 1 ml. each. The first samples were taken one minute after the completion of the injection, the others 30, 60, 120, and 240 minutes thereafter. Aliquots of plasma (0.1 cc.) were then taken for counting. At the end of the experiment the animals were killed by asphyxiation. The brain and the hypophysis were removed separately, the latter being divided into its anterior and posterior lobes. All these parts were then hardened in a 10% formalin solution for 24 hours. After hardening, the brain was divided as shown in Figure 1 and the parts were blotted dry, weighed on a torsion balance, and placed in shell vials, with fresh formalin solution. The tissues were then counted, in the vials, in a well-type scintillation counter. The tissues from the pituitary lobes and those from the pineal gland were counted in the formalin in which they were fixed.

Some of the experiments were carried out with labelled triiodothyronine, others with labelled thyroxine. Identical experiments were done with radioiodinated human serum albumin (RISA) as the injected material instead of one of the hormones, in order to establish the approximate size of the plasma space in the various tissues. The data

[2] This is a dose of approximately 23.7 μc./kg. for triiodothyronine and of approximately 10.0 μc./kg. for thyroxine. Since the specific activity of each shipment varies somewhat, the amount of radioactivity in a given dose of thyroid hormone will vary.

obtained from the RISA experiments were used to determine more precisely the amount of radioactivity in the plasma of the tissue samples taken from animals which had received a labelled thyroid hormone. As has been stated, the dose of hormone in a typical experiment was 0.5 μg./kg. (23.7 μc./kg.). In one series of experiments with thyroxine a larger dose, consisting of 4 μg./kg., was given. Experiments on single animals which had been given large doses (4.0 μg./kg. and 12 μg./kg., respectively) of labelled triiodothyronine and examined according to the methods described here had been done for other purposes than those of this study, but pertinent data from these experiments are included in this report wherever they apply.

A series of autoradiographic experiments was also performed, in an attempt to define

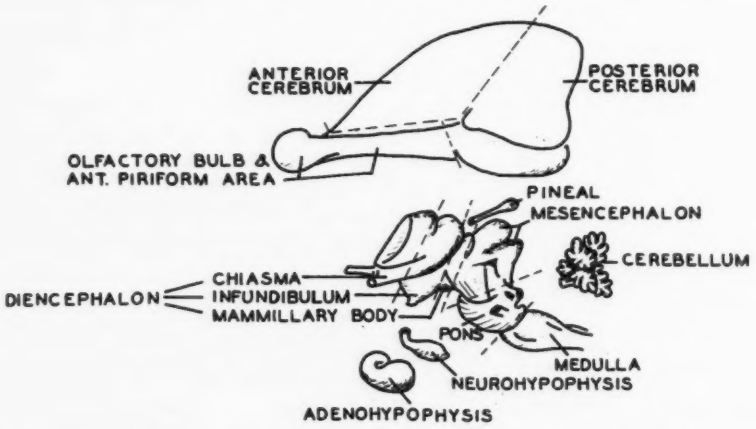


FIG. 1. An exploded view of the rabbit brain, showing the various parts which were studied for their content of total radioactivity after the injection of I^{131}-labelled thyroid hormones.

more clearly than was possible in the other experiments the areas in which the radioactive hormones were concentrated. In order to insure the localization in the brain of a high enough intensity of radioactivity to produce autoradiographs, very large doses of labelled triiodothyronine were used, each of two rabbits receiving approximately 500 μc./kg. (10 and 11 μg./kg., containing 475 and 520 μc./kg., respectively.) One of the animals was killed 2 hours after the injection, the other 4 hours. The brains were removed and fixed in a 10% formalin solution. In the second animal, the brain was first perfused free of blood. Frozen sections from both brains were cut at 25μ and mounted on slides previously coated with a 1% gelatin solution, After the slides had dried, they were dipped into a solution of 1% celloidin in a 50:50 ether-alcohol mixture and allowed to dry again. The slides were then applied to Kodak no-screen x-ray film in an x-ray film holder and left to be exposed for periods varying from 6 to 48 hours. For a negative control, similar preparations were made from the brain of a non-injected animal.

In some experiments, in which a dose of 0.5 μg. or of 4.0 μg. of triiodothyronine or thyroxine was given, chromatographic analysis of plasma samples and of unfixed pituitary and brain tissue was done. Aliquots of whole plasma or distilled water homogenates of fresh tissue were analyzed on ascending paper chromatograms. The solvent used was n-butanol-dioxan-ammonia. After development of the chromatogram with ninhydrin and palladium chloride, the chromatographic strips were scanned under a gas-flow count-

er. The peaks obtained were related to the location of the known carrier substances, nonradioactive triiodothyronine, thyroxine, and iodide. The areas corresponding to the radioactivity peaks of the different I^{131}-containing substances were cut from the paper and counted. The resulting data were then expressed as percentages of the total activity present on the chromatogram and indicate the relative composition of the sample analyzed. If the samples to be studied contained low concentrations of radioactivity they were extracted with either n-butanol or ethanol. The procedure consisted of three extractions: the first with a double volume of solvent, and the succeeding two with solvent volumes equal to the volumes of the material being extracted. These extractions resulted in the recovery of over 85% of the tissue radioactivity. The alcohol fractions were pooled and reduced in volume under low pressure at room temperature. The concentrated solution was then applied to the paper for analysis and studied according to the procedure followed with the non-extracted tissue.

RESULTS

In the process of fixing the tissues in formalin, some of the radioactivity which they had contained when the animals were killed escaped into the formalin solution. This activity was identified as iodide, by chromatographic analysis of the formalin solution. The brain lost only 2.5% of its radioactivity in this manner, but the pituitary and the pineal glands lost from 20 to 25%. Since the tissues from the pituitary lobes and the pineal gland were counted with the formalin in which they were fixed, the counts obtained for these locations reflect the total amount of radioactive substance present in these tissues when the animals were killed. The counts for the brain tissue, however, are based on only 97.5% of the radioactivity which was present when the animals were killed, as the brain tissues were covered with fresh formalin after they had been divided.

Early in the triiodothyronine experiments designed for autoradiographic analysis, it was noted that formalin appeared to enhance the binding of thyroid hormone to the tissue protein. Chromatographic analysis of formalin-fixed and of fresh pituitary homogenates (Fig. 2) showed that all the radioactivity in the fixed preparation remained at the origin, while the radioactivity in the fresh tissue migrated in the expected manner, in this instance to the position occupied by carrier triiodothyronine. In control experiments, both ethanol and butanol were relatively ineffective in extracting the organic iodine compounds from fixed tissues. The iodine could, however, be extracted if the fixed tissues were dissolved in NaOH and acidified to pH 3.5. This suggested that formalin fixation immobilized and bound the injected thyroid hormone to the tissue in which it was localized. No losses from the section were detected during the sectioning and mounting procedure. It was, therefore, felt that the foci of radioactivity revealed by the autoradiographic procedure represent, in all probability, the actual sites of localization of triiodothyronine just prior to the death of the animal.

The Distribution of Total Radioactivity in the Brain and the Hypophysis

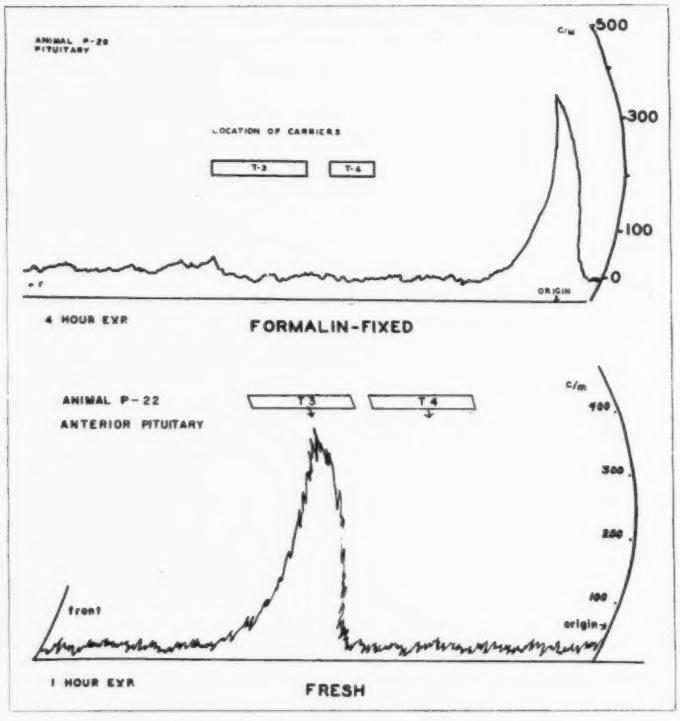


FIG. 2. The chromatographic separation of I^{131} in fresh and formalin-fixed rabbit pituitary tissue after intravenous injection of I^{131}-labelled triiodothyronine. T_3 = triiodothyronine; T_4 = thyroxine; F = front; and C/M = counts per minute.

After Administration of Triiodothyronine. After intravenous injection of I^{131}-labelled triiodothyronine, the radioactive material was rapidly cleared from the plasma (Fig. 3). This clearance was most rapid in the first 30 minutes of the experiment, during which 90% of the injected material left the circulation. After this initial removal, the concentration of plasma I^{131} tended to level off and to fall more slowly (Figs. 3 and 4). These changes were not reciprocally reflected by the entry of radioactivity into the tissues. Instead, the I^{131} concentration in both pituitary lobes rose relatively slowly and reached a maximum only after about two hours. It then decreased slowly during the remainder of the experiment (Fig. 3). The mean radioactivity of the diencephalon and the remaining brain (Fig. 4) reached a peak at one hour and tended to maintain this concentration despite a falling plasma radioactivity. This pattern of uptake was reflected in the various areas of the brain studied in the experiments (Table 1). There is a suggestion that the decline of radioactivity between the time of peak activity and the fourth hour of the experiment was slowest in the diencephalon

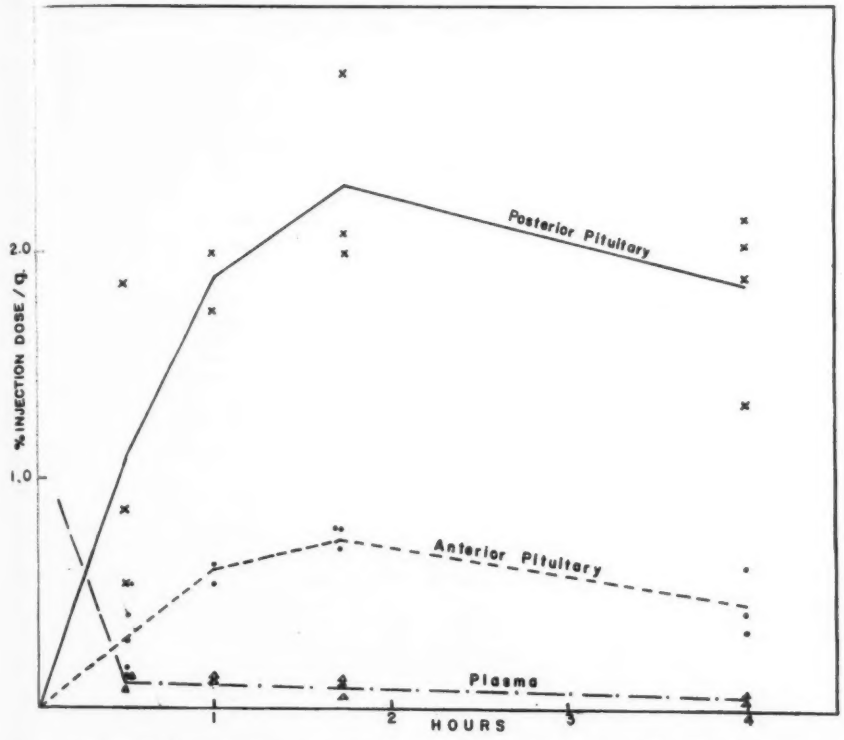


FIG. 3. The concentration of I^{131} in the pituitary and plasma after the intravenous injection of 0.5 μg./kg. of triiodothyronine at various time intervals. The crosses, dots, and triangles indicate individual values—crosses for the posterior pituitary lobe, dots for the anterior pituitary lobe, and triangles for the plasma.

TABLE 1. THE CONCENTRATION OF TOTAL RADIOACTIVITY IN THE BRAIN OF THE RABBIT AT VARIOUS TIMES AFTER THE INTRAVENOUS INJECTION OF 0.5 μG./KG. OF I^{131} LABELLED TRIIODOTHYRONINE

Time	30 minutes	60 minutes	105 minutes	240 minutes
No. of animals	3	2	3	4
	% dose/gm.	% dose/gm.	% dose/gm.	% dose/gm.
Anterior Cerebrum		0.035	0.042*	0.026
Posterior Cerebrum	0.009	0.035	0.040	0.028
Olfactory Area		0.034	0.034*	0.024
Cerebellum	0.011*	0.043	0.041	0.031
Chiasmic Diencephalon	0.015	0.032	0.039	0.029
Tuberal Diencephalon	0.020	0.038	0.041	0.035
Mammillary Diencephalon	0.020	0.037	0.035	0.035
Mesencephalon	0.012*	0.039	0.039	0.028
Pons	0.012*	0.038	0.037	0.032
Medulla	0.010*	0.038	0.033	0.028

* Value from only one animal.

(Fig. 4), particularly in the mammillary body, and was perhaps most rapid in the cerebrum (Table 1).

In some cases the gray and the white matter of the cerebrum were separated and the radioactivity measured. The gray matter was found to contain about twice as much radioiodine as the white. In order to test this apparent relationship between concentration of radioactivity and gray

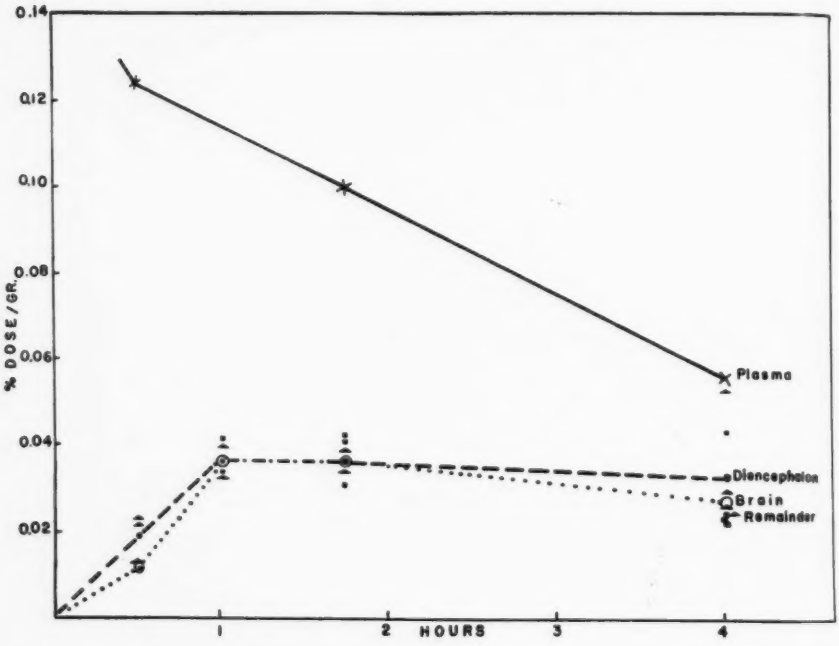


FIG. 4. The concentration of I^{131} in the diencephalon, brain remainder, and plasma after the intravenous injection of I^{131}-labelled triiodothyronine at various time intervals. The crosses, dots, and hollow circles indicate the mean values for plasma, diencephalon, and brain remainder, while the triangles (diencephalon) and squares (brain remainder) indicate individual values.

matter content, the autoradiographic studies previously described were undertaken. Since the size of the dose had been found to have no significant effect on the pattern of distribution of the hormone (Table 2), it was thought safe to conclude that even with the high doses of triiodothyronine used to produce autoradiographs (10 and 11 μg./kg., respectively) a distribution would be achieved corresponding to that produced by the 0.5 μg./kg. doses given in the typical experiments. The autoradiographic results are shown in Figure 5 (Sections 1–12). Perfusion of the brain in one animal did not alter the pattern of distribution of the radioactivity; nor

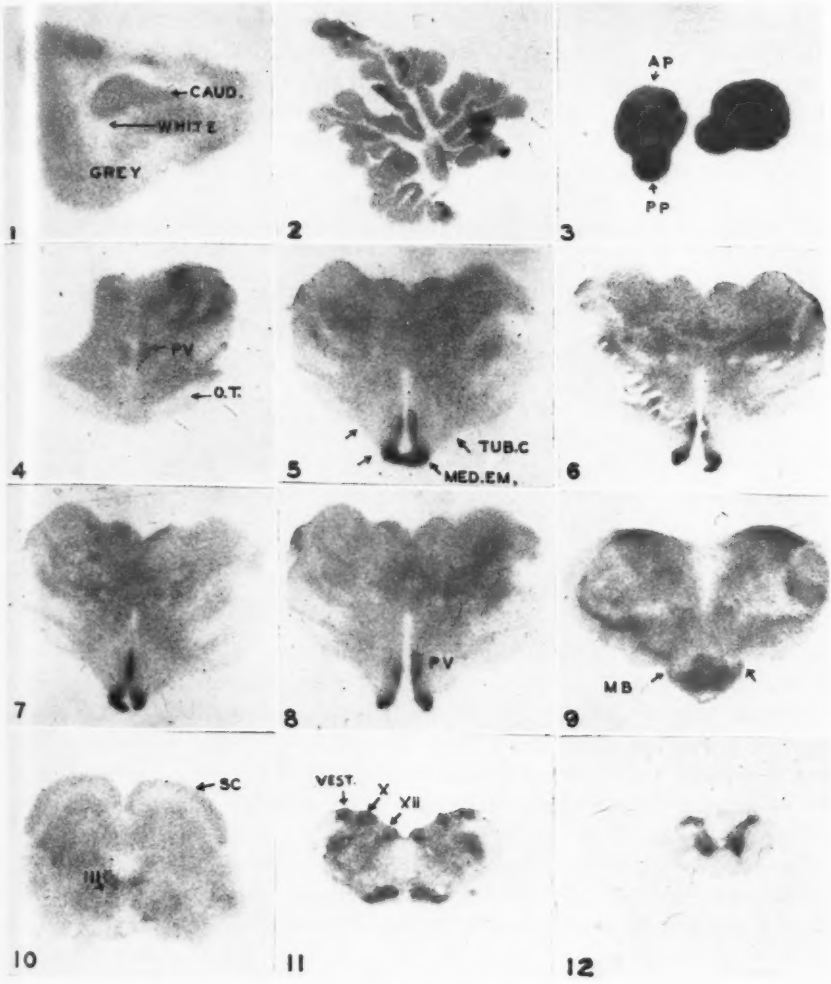


FIG. 5. An autoradiographic representation of the sites of localization of I^{131} in the rabbit brain after injection of I^{131}-labelled triiodothyronine: 1, Coronal section of the cerebrum; 2, Sagittal section through the vermian cerebellum; 3, Pituitary; 4, Chiasmic diencephalon; 5–8, Coronal sections through the tuberal diencephalon; 9, Coronal sections through the diencephalon at the mammillary body; 10, Coronal section through the superior colliculus; 11, Coronal section through the open medulla oblongata; and 12, Coronal section through the spinal cord. AP = anterior pituitary; Caud. = caudate nucleus; SC = superior colliculus; Tub.C. = tuber cinereum; Vest. = vestibular nuclei; X = vagal nuclei; and XII = hypoglossal nucleus.

was there loss in activity due to dipping of the slides in the cellodin-ether-alcohol mixture. The results indicate that the radioactivity concentrated in the brain was predominantly associated with the nuclear areas. The heaviest concentrations in the brain occurred in the diencephalon, in the areas occupied by the median eminence and the paraventricular nucleus (Fig. 5, Sections 5–8), but the most positive autoradiographic reactions occurred over the neurohypophysis and, somewhat less positive, over the adenohypophysis (Fig. 5, Section 3). Chromatographic analysis of the brain tissue showed that the radioactivity was predominantly in the form of triiodothyronine (Fig. 6). Thus, these autoradiographs represent the localization of triiodothyronine in the brain. Since the control preparations, after a 168-hour exposure, did not produce any blackening of the film, the possibility of artifactitiously produced autoradiographs through chemical action of the section on the emulsion can be ruled out.

TABLE 2. THE DISTRIBUTION OF RADIOIODINE IN THE RABBIT BRAIN AND PITUITARY FOUR HOURS AFTER VARIOUS DOSES OF LABELLED TRIIODOTHYRONINE

	% dose/gm.		
	0.5 μg./kg.	4 μg./kg.	12 μg./kg.†
No. of animals	4	4	1
Plasma	0.056	0.057	0.045
Anterior Pituitary Lobe	0.47	0.38*	0.26
Posterior Pituitary Lobe	1.86	2.07*	1.52
Diencephalon	0.032	0.041	0.053
Cerebrum	0.026	0.042	0.053

* Values from two animals only.
† Given in three divided doses of 4 μg./kg. each, at half-hour intervals from the start of the experiment.

Comparison of the Distribution of Labelled Thyroxine and Triiodothyronine. In the first of the two series of experiments with labelled thyroxine, three animals were injected with a dose of the same size as that used in the typical triiodothyronine experiments and were killed about 105 minutes after injection. This interval was the period during which maximal uptake had occurred after triiodothyronine injection (see Fig. 3). The rates of uptake of the two substances during this period are shown in Table 3.

Here it is seen that the mean level of radioactivity in the plasma of the thyroxine-treated animals was about six times as high as in those treated with triiodothyronine. Despite this, the concentration of radioiodine in the pituitary and various parts of the brain after thyroxine injection was only a third to a half as great as that found after administration of an equivalent dose of triiodothyronine. Thus, the tissue-to-plasma concentration gradients were from twelve to eighteen times greater in the triiodothy

rone-treated animals (Table 3) than in those treated with thyroxine. It was thought possible that these differences were due to a slower tissue-uptake after thyroxine. That this was unlikely for the brain is shown by

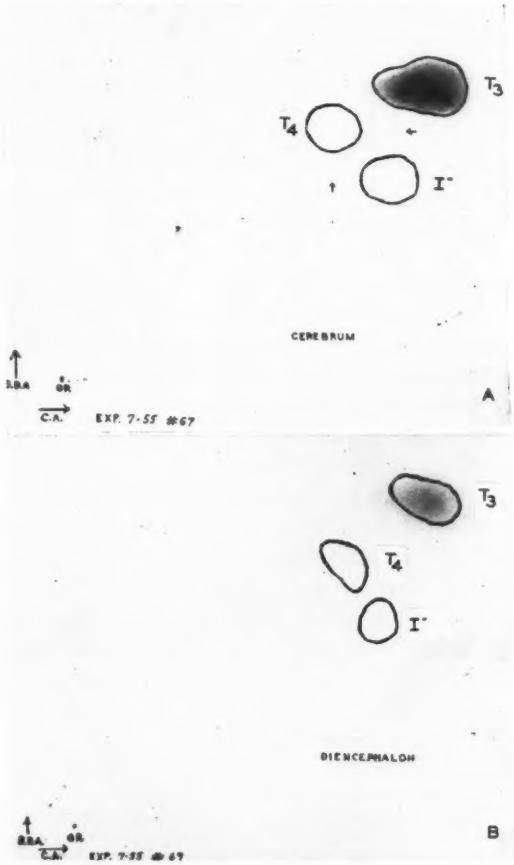


FIG. 6. Two-dimensional chromatogram showing the I^{131}-containing compounds present in the cerebrum (A) and diencephalon (B) after intravenous injection of 4 μg./kg. of I^{131}-labelled triiodothyronine. BDA = n-butanol-dioxane-ammonia, Solvent 1; C.A. = collidine-ammonia, Solvent 2; T_3 = triiodothyronine; T_4 = thyroxine; I^- = iodide. The arrows in A indicate the location of two spots which were visible on the x-ray film. The position of these spots does not conform to that of any of the known metabolites of the thyroid hormone.

the results of the second series of experiments with thyroxine (Table 4), in which for purposes of biochemical analysis a larger dose had been injected. The data show that the I^{131}-uptake in parts of the brain after injection of 4.0 μg./kg. of thyroxine had reached a level at one hour which re-

TABLE 3. A COMPARISON OF THE DISTRIBUTION OF TISSUE RADIOACTIVITY 105 MINUTES AFTER INTRAVENOUS ADMINISTRATION OF 0.5 μG./KG. OF LABELLED TRIIODOTHYRONINE OR THYROXINE

Animal No.	T_3* % dose/gm.					T_4* % dose/gm.					Ratio of concentration T_3* to T_4*
	P3	P5	P7	Mean	o/p†	P11	P12	P14	Mean	o/p†	
Plasma	0.10	0.10	0.11	0.10		0.54	0.22	1.22	0.66		0.15
Anterior Pituitary Lobe	0.79	0.69	0.76	0.75	7.5	0.31	0.11	0.36	0.26	0.39	2.9
Posterior Pituitary Lobe	2.00	2.10	2.81	2.30	23.0	0.61	0.59	1.46	0.89	1.34	2.6
Cerebrum	0.042		0.040	0.041	0.41	0.019	0.005	0.019	0.014	0.021	2.9
Cerebellum	0.043	0.033	0.048	0.041	0.41	0.019	0.008	0.024	0.017	0.026	2.4
Chiasmic Diencephalon	0.031	0.032	0.035	0.033	0.33	0.021	0.005	0.022	0.016	0.024	2.1
Tuberal Diencephalon	0.042	0.037	0.044	0.041	0.41	0.020	0.004	0.022	0.015	0.023	2.7
Mammillary Diencephalon	0.036	0.033	0.036	0.035	0.35	0.020	0.004	0.027	0.017	0.026	2.1
Mesencephalon	0.041	0.034	0.042	0.039	0.39	0.014	0.005	0.021	0.013	0.020	3.0
Pons	0.041	0.031	0.039	0.037	0.37	0.015	0.006	0.023	0.015	0.023	2.5
Medulla	0.038	0.025	0.045	0.036	0.36	0.016	0.008	0.018	0.014	0.021	2.6

* T_3 and T_4 indicate Triiodothyronine and Thyroxine, respectively.
† o/p indicates organ plasma ratio.

mained more or less constant over the subsequent three hours and was of the same order of magnitude as the uptake after 0.5 μg./kg. of thyroxine (Table 3). In the pituitary lobes, after the larger dose of thyroxine (Table 4) the peak uptake would appear to have occurred between the first and second hours after the administration of the dose, after which its level remained constant. The level of concentration of triiodothyronine had shown a slow decline after reaching its peak at two hours (Fig. 3). However, the rate at which both hormones achieve their peak tissue concentration appears to be similar. It would seem, then, that after equivalent doses of triiodothyronine and thyroxine (0.5 μg./kg.), the shapes of the uptake curves to the peak point, though not the level of peak concentration, are sufficiently similar to substantiate the comparison shown in Table 3. However, while peak concentration in the tissues was achieved in the same period of time by the two hormones, the rate of metabolic degradation was apparently faster for triiodothyronine than for thyroxine.

TABLE 4. THE DISTRIBUTION OF RADIOIODINE IN THE RABBIT BRAIN AND HYPOPHYSIS AT VARIOUS TIMES AFTER INTRAVENOUS ADMINISTRATION OF 4 μG./KG. OF LABELLED THYROXINE

Time	60 minutes	120 minutes	240 minutes
No. of animals	1	1	1
	Percentage of the dose per gram		
Plasma	0.49	0.32	0.34
Anterior Pituitary Lobe	0.11	0.13	0.14
Posterior Pituitary Lobe	0.33	0.58	0.58
Chiasma Diencephalon	0.008	0.005	0.015
Infundibular Diencephalon	0.014	0.009	0.014
Mammillary Diencephalon	0.012	0.006	0.014

The Effect of the Size of the Dose on Uptake. As was noted above, there was little difference in the percentage of the dose taken up (cf. Tables 3 and 4) after the injection of various doses of thyroxine. A similar result was noted in the case of triiodothyronine when doses of 0.5, 4.0, and 12.0 μg./kg. were compared (Table 2). The range of individual values was large enough that any differences seen in the means would not be statistically significant. It is evident that, within the limits of dosage used, the amount of either thyroxine or triiodothyronine taken up at the various sites was proportional to the levels of the two hormones in their respective metabolic spaces. Thus, the capacity of a tissue to concentrate more activity after triiodothyronine than after thyroxine was not affected by the dose level.

The Chemical Nature of the Radioiodine in the Tissues. The chemical nature of the radioactivity in the tissues and the plasma was examined by paper chromatography. The results of these examinations are illustrated in Figures 7 and 8. In these figures, the fraction labelled "residue" represents total activity measurable in those parts of the paper which remained after the areas with known carrier substances had been removed. In the chromatographic scans this residue activity was not seen as a discrete peak but was diffusely distributed throughout the paper.

From Figure 7 it may be seen that after injection of triiodothyronine the proportion of the plasma I^{131} identifiable as triiodothyronine steadily decreased as the experiment proceeded, reaching an average level of 45%. This decrease was accompanied by an increase in the proportion of radioiodine present as iodide. In the pituitary, on the other hand, about 90% of the I^{131} in both lobes was present as triiodothyronine, and this high level was maintained in hypophyses sampled over the four-hour experimental period. Similarly, the cerebrum and the diencephalon at four hours after triiodothyronine injection contained predominantly triiodothyronine (Fig. 6). In the chromatogram of cerebral tissue two faint spots were noted in the areas indicated by the arrows. These do not correspond to the locations of any of the known triiodothyronine derivatives and are clearly remote from the localization of its acetic and propionic analogues.

After thyroxine injection, the plasma radioactivity was predominantly in the form of thyroxine (Fig. 8) and constituted over 88% of the activity present during the course of the experiment. In the hypophysis, between 60 and 80% of the radioiodine was identified as thyroxine and between 6 and 20% as triiodothyronine. While chromatograms of the one-minute plasmas of thyroxine failed to show any peak in the triiodothyronine area, it is possible that some contaminant triiodothyronine may have been the precursor of that found in the pituitary gland.

From the intensity of the spots in Figure 6 and from other unpublished analytical data, it was estimated that between 90 and 96% of the radioiodine concentration in the brain after injection of triiodothyronine or

thyroxine could be identified as the particular iodothyronine injected. From these percentages and those shown above for the pituitary it was possible to calculate the percentage of the dose present as either thyroxine or triiodothyronine. At 105 minutes after the injection of triiodothyronine, an average of 50% of the plasma radioactivity was identifiable as triiodo-

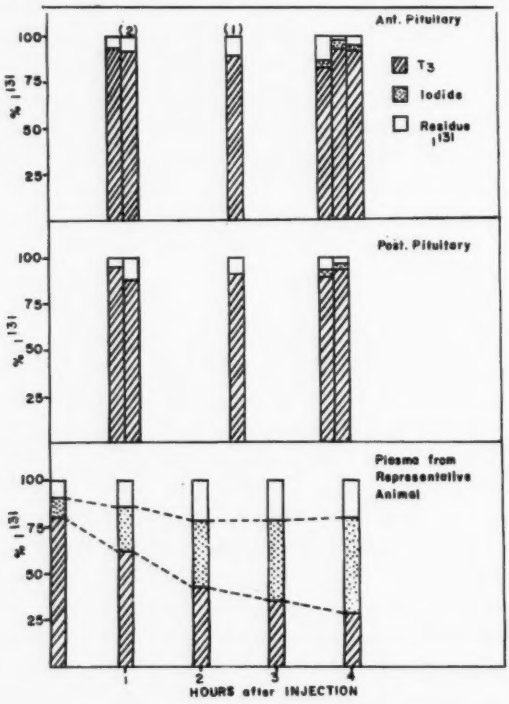


FIG. 7. A graphic representation of the chromatographic analysis of the various metabolites of I^{131}-labelled triiodothyronine in the pituitary and plasma. The residue is that fraction of radioactivity measurable in the parts of the chromatogram which remains after the areas with known carrier substances have been removed. The dosage for all animals represented here was 0.5 μg./kg. except in two cases, for which the dose in μg./kg. is indicated by the numbers in parenthesis at the tops of the columns.

thyronine. At this time, the concentration of total radioactivity was 10% of the dose per gram (cf. Table 3). The concentration of triiodothyronine in the plasma would then be $50/100 \times .10 = .05\%$ of the dose per gram. The data obtained in this way are given in Table 5. In addition, concentration gradients were obtained by calculating the ratio of the concentration in an organ to the concentration in the plasma at the same time (organ/plasma ratio). Ratios thus obtained are also included in Table 5 and are compared with similar ratios calculated after the injection of doses of radioiodinated human serum albumin (RISA). The latter ratios were expected to give an

estimate of the proportion of the organ radioiodine which could be attributed to plasma remaining in the sample.

Analysis of the figures in Table 5 indicates the following points: (*1*) High concentration ratios of triiodothyronine were maintained by both the pituitary gland and the brain, and the gradients thus produced tended to become steeper with time (cf. triiodothyronine o/p ratios after 0.5 μg./kg. at 105 and 240 minutes shown in Table 5). The slopes of the gradients at 105 and 240 minutes indicate that uptake was more rapid than degrada-

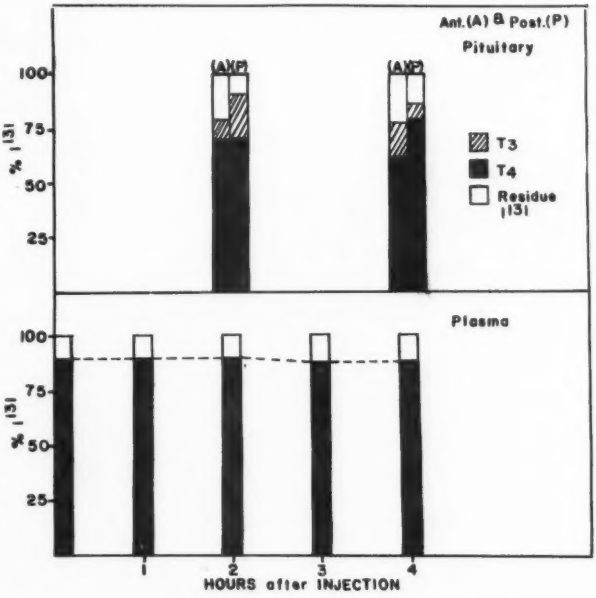


FIG. 8. A graphic representation of the chromatographic analysis of the various metabolites of I^{131}-labelled thyroxine in the pituitary and plasma after a dose of 4 μg./kg. Residue is calculated as in Figure 7.

tion. The gradients were of much the same slope when an eight-times-as-large dose of triiodothyronine was injected (cf. ratios at 240 minutes after doses of 0.5 μg. and 4 μg./kg., respectively). (*2*) After thyroxine, the concentration ratios were considerably lower, being only 1/50th and 1/30th the ratios of triiodothyronine in the pituitary gland and the brain, respectively (Table 5), and their gradients showed much less elevation with increased time. This suggests that the rate of uptake and degradation may be more nearly balanced than with triiodothyronine, uptake only slightly exceeding degradation. The highest concentration occurred in the neurohypophysis. In the other tissues the ratios, while rather low, exceeded those obtained after RISA injection. Thus, the values were not a reflection of the plasma radioactivity which might have remained in the samples at

the time of preparation, but indicated, rather, the movement of thyroxine into an extravascular space. The extent of this space is considerably less than the extracellular space, if one accepts for the water space in the brain the reported ratios for radioactive sodium and chloride of 0.11 at one hour and 0.33 at 12 hours after injection.

Miscellaneous Observations. From calculations similar to those outlined above, it can be shown that at four hours there was a concentration of

TABLE 5. THE DISTRIBUTION OF CHROMATOGRAPHICALLY IDENTIFIABLE TRIIODOTHYRONINE OR THYROXINE AT VARIOUS TIMES AFTER VARIOUS INJECTED DOSES

(A) At 105 minutes after injection

	T_3* 0.5 μg./kg.		T_4* 0.5 μg./kg.		Ratio of concentration of T_3 to T_4	RISA‡ o/p†
	% dose/gm.	o/p†	% dose/gm.	o/p†		
Plasma	0.050	1	0.58	1	0.086	1
Anterior Pituitary Lobe	0.67	13	0.18	0.31	3.72	0.08
Posterior Pituitary Lobe	2.06	41	0.62	1.1	3.33	0.09
Diencephalon	0.034	0.7	0.015	0.03	2.27	0.004
Cerebrum	0.039	0.8	0.013	0.02	3.0	0.003

(B) At 240 minutes after injection

	T_3* 0.5 μg./kg.		T_3* 4 μg./kg.		T_4* 4 μg./kg.		RISA‡ o/p†
	% dose/gm.	o/p†	% dose/gm.	o/p†	% dose/gm.	o/p†	
Plasma	0.025	1	0.025	1	0.30	1	1
Anterior Pituitary Lobe	0.42	17	0.34	14	0.10	0.33	0.09
Posterior Pituitary Lobe	1.67	67	1.86	75	0.41	1.4	0.09
Diencephalon	0.030	1.2	0.039	1.5	0.013	0.04	0.006
Cerebrum	0.025	1	0.040	1.6	0.013	0.04	0.004

* T_3 and T_4 indicate Triiodothyronine and Thyroxine, respectively.

† o/p indicates the organ to plasma ratio. $\left(\frac{\%\text{ Dose/gm. organ}}{\%\text{ Dose/gm. plasma}}\right)$.

‡ The RISA (radio iodinated serum albumin) o/p ratios are included to indicate the relative size of the plasma space as compared with the thyroid hormone spaces.

iodide in the neurohypophysis and possibly also in the adenohypophysis after triiodothyronine administration. The o/p ratios were about 1.5 for the posterior lobe and 0.65 for the anterior lobe.

In a single animal, the pineal gland, the superior cervical ganglion, and the cerebrospinal fluid were examined four hours after injection of triiodothyronine (8.0 μg./kg.). The values obtained were 0.067, 0.044, and 0.001% of the injected dose, respectively. The corresponding organ-plasma ratios were 1.9, 1.3, and 0.02. The very low concentration of radioactivity in the cerebrospinal fluid has been a consistent finding in other, unpublished, experiments with other species, and the source of the activity has been identified chromatographically as iodide.

DISCUSSION

The results presented here may be best discussed in relation to the metabolism of endogenously formed thyroid hormone by the rabbit hypophysis and the brain. Information on this subject has been obtained from experiments in which tracer radioactive iodide is administered to label the substrate pool for hormonogenesis. The endogenous hormone thus labelled can then be detected as organic iodide, and its accumulation by the tissues under investigation can be followed. Iodide itself does not appear to be concentrated by rabbit pituitary or brain at either two hours (4) or four hours (5). At later times (6), a concentration of radioactivity in the hypophysis can be demonstrated. It disappears after thyroidectomy. Similar findings have been reported for the rat and the guinea pig (7). Recently Sloviter and Morel (8) have further clarified this metabolic process by showing in iodine-deficient rabbits that 48 hours after injecting radioiodide there was a concentration of organic iodine in both the posterior and anterior hypophyseal lobes, the organ/plasma ratios being 3.2 (6.2 to 1.3) and 0.8 (1.4 to 0.50), respectively. Moreover, they demonstrated a concentration of iodide in the posterior lobe. The conclusion from these experiments would be that the thyroid hormone is concentrated in the posterior hypophysis at least and is deiodinated there.

It may be assumed that the predominant hormonal substance in the blood is thyroxine, and that the organ/plasma ratios for the hypophysis obtained after the administration of 0.5 μg./kg. of radioactive thyroxine (Table 3) should approximate the values for the endogenously-formed hormone. The values after injection of labelled thyroxine were 1.3 for the posterior and 0.4 for the anterior lobe. Courrier *et al.* (9) reported values of 3.2 and 0.4 after a dose of about 1.3 μg./kg. of labelled thyroxine at the same time interval after injection (120 minutes), while Taurog *et al.* (10), in their time study after administration of about 20 μg./kg. of thyroxine, obtained roughly equivalent ratios up to thirty hours after injection. While most of the concentration ratios for the posterior lobe after thyroxine injection fall within the range of the concentrations of the endogenous hormone (6.2 to 1.3), the endogenous hormone ratios for the anterior lobe (1.4 to 0.5) indicate a concentration of hormonal iodine greater than that after exogenous thyroxine administration (0.4). This concentration might be explained on the assumption that part of the endogenous hormonal iodine in the plasma is present as triiodothyronine, a not unlikely possibility in an iodine-deficient animal. It is more likely, however, that most of the organic iodine found in the pituitary gland during metabolism of the endogenous thyroid hormone is present as thyroxine.

There has been some question as to whether there is any entry of thyroxine into the anterior lobe of the hypophysis. In the experiments reported here, it was thought that the small concentrations of thyroxine found in the adenohypophysis might be attributable to the plasma trapped within

the tissue when the animals were killed. However, when the plasma content was determined through the use of radioiodinated serum albumin (Table 5), the thyroxine space was found to be about four times as large as the plasma space. It is evident, therefore, that there is an extravascular compartment for thyroxine in the adenohypophysis. It is not certain to what extent this space is intracellular, although from the autoradiographs of Jensen and Clark (5)[3] and from similar studies in the guinea pig (Ford and Gross, unpublished), it would appear to be diffusely distributed over the parenchyma of the gland. While the thyroxine space is usually equivalent to or perhaps a little smaller than the sodium space (cf. Results, this paper, and Courrier *et al.*, 9), it does not necessarily follow that thyroxine is distributed predominantly in the interstitial areas. Circulating thyroxine is bound to plasma proteins (11) and, being non-dialyzable, would not be expected to be distributed in the tissues in the same pattern as a freely diffusible ion. Further, the level of thyroxine concentration in this space is roughly proportional to the level of plasma thyroxine, since the percentages of the dose present at two hours after administration of either 0.5 or 4 μg./kg. are similar; that is, 0.18% and 0.13%, respectively (see Tables 4 and 5). Rough calculations from the data of Taurog *et al.* (10, Table 4) would indicate that this is also the case with higher dosages. In view of the demonstration that thyroxine injected directly into the anterior lobe of the rabbit pituitary gland affects the production of thyroid-stimulating hormone (TSH) (12), and in view of the well-established quantitative relationship between thyroxine levels of the circulating plasma and TSH production, it is likely that the thyroxine space, or compartment, although relatively small, is of biological importance.

In following the metabolism of thyroxine by the pituitary gland, it was possible to demonstrate the presence of triiodothyronine in both lobes (Fig. 8). This triiodothyronine could have been produced by the deiodination of thyroxine, a conjecture which would substantiate the findings of Sloviter and Morel (8)[4] for the posterior lobe. The other possible source could be the plasma, where triiodothyronine might have been present as (a) a contaminant of the injected thyroxine, or (b) a thyroid secretion, or (c) a product of the deiodination of thyroxine elsewhere in the body. The

[3] It is likely that the preparations of radioactive thyroxine used by Jensen and Clark contained some contaminant triiodothyronine, since, at the time when these experiments were carried out, the purification methods available did not affect the separation of the two compounds. In view of this possibility and the greater concentration of triiodothyronine by neural tissues, it may be that some of the autoradiographic reaction in the hypophysis of nervous tissue illustrated by these authors was due to triiodothyronine.

[4] It is likely that in the experiments of Sloviter and Morel there was also some concentration of iodide in the anterior lobe of the pituitary after the course of the metabolism of endogenously-formed thyroid hormone, since they reported organ/plasma ratios for inorganic iodide of 0.62 (from 0.44 to 0.85) as against ratios of 0.3 (from 0.26 to 0.30) after radioactive iodide injection, as given in the paper by Taurog *et al.* (8).

first two suggested sources of plasma triiodothyronine seem unlikely, since triiodothyronine could not be detected on the chromatograms of the plasma samples. The third source cannot be ruled out at the present time; we may be concerned here with a transport form of triiodothyronine with a chromatographic migration different from that of free triiodothyronine, the existence of which has been suggested (13).

The findings on the distribution and metabolism of triiodothyronine represent an extension and elaboration of preliminary work reported earlier by the authors (14, 15) and are in agreement with similar studies by Courrier *et al.* (9) and Taurog *et al.* (10). The shape of the uptake curves (Figs. 3 and 4) may reflect a slow rate of concentration in a large tissue pool with a slow turnover. There is, however, evidence that a triiodothyronine complex is formed in the liver, and perhaps in the kidney, and that it may be secreted back into the circulation, where it constitutes the precursor of the triiodothyronine concentrating in the other body tissues. The curve of appearance of this substance in the blood (13) bears a good precursor relationship to the uptake curves shown in Figures 3 and 4.

With further regard to the metabolism of triiodothyronine, it is clear from Figures 6 and 7 that triiodothyronine itself is concentrated in the hypophysis and the brain. The concentration of iodide in both lobes of the pituitary would indicate that deiodination of triiodothyronine takes place at these sites. (See the discussion on the deiodination of endogenous hormone given above.) In the cerebrum and the diencephalon (Fig. 6) the predominant radioactive component is triiodothyronine. There were insufficient quantitative chromatographic data to determine whether or not deiodination occurred in these locations. Unpublished experiments on guinea pig brain *in vivo*, however, and the results obtained by Tata and his associates (16) on brain slices *in vitro* would indicate that deiodination probably does occur. It may be noted that none of the metabolic products (i.e., deaminated iodothyronines) found by Tata *et al.* in tissue homogenate systems containing triiodothyronine could be detected on our chromatograms. It is possible, as suggested by these authors, that the intermediates were rapidly deiodinated by intact nervous tissue and that, if present in our material, could not, therefore, be detected. Pending evidence that the deaminated iodothyronines are rapidly deiodinated *in vivo* by the brain or by brain slices *in vitro*, the metabolic sequence for triiodothyronine and thyroxine suggested by Tata *et al.* must for the moment be considered hypothetical. Our chromatograms did indicate the presence of two unknown substances (see arrows in Fig. 6A).

In comparing the distribution of equal doses of thyroxine and triiodothyronine, it is interesting to note that ratios of the concentration of triiodothyronine to that of thyroxine are rather similar in all the tissues tested. On the basis of total iodine concentration (Table 3), this ratio is about 2.5. When it is calculated on the basis of the iodothyronine concen-

tration (from data in Table 5, A) the ratio exceeds 3. These ratios are of the same order as the relative biological potency of the two substances and may account in part for the quantitative difference in their biological activities.

As to the biological significance of the localization of these hormones, the concentration of triiodothyronine in the hypophysis, median eminence, and paraventricular nucleus (Fig. 5) may be related to the hypothalamo-hypophyseal regulatory mechanism for the secretion of pituitary hormones. It is well established that hypothalamic centers regulate thyroid activity. Thus, section of the pituitary stalk, transplantation of the pituitary gland to the anterior chamber of the eye, or destruction of anterior hypothalamic centers reduces thyroid function, probably through a reduction in TSH output. (For a review of the literature on this point refer to Brown-Grant, 17.) It has been suggested (17) that concentrations of thyroid hormone in the hypothalamus regulate the level of thyroid hormone in the hypophyseal portal vessels, which in turn would modify the thyroid hormone content of the blood to which the adenohypophyseal cells are exposed. It is difficult to reconcile this hypothesis with the findings reported here. It seems unlikely that the large concentration of triiodothyronine by the adenohypophysis (cf. Table 5) would be significantly affected by accumulations occurring in the hypothalamus (see radioautographic results in Figure 5 and the concentration data in Table 3). A further requirement of the aforementioned hypothesis would be the rapid degradation or turnover of hormone in the hypothalamus in order to maintain a steady state. The curves in Figure 4 do not indicate a rapid turnover, and the chromatographic analysis (Fig. 6) does not indicate a rapid degree of degradation. A more likely hypothesis would be that the concentration of thyroid hormone in the hypothalamus is associated with the production or release of some neurosecretory agent which stimulates TSH release. This would be analogous with the hypothalamic factor apparently involved in ACTH release (Guillemin and Rosenberg, 18). In this connection, too, it is possible that the hypothalamic concentration of triiodothyronine represents the site at which triiodothyronine acts to stimulate the production of ACTH (Eartly and Leblond, 19).

However, von Euler and Holmgren showed that injections of thyroxine into the hypothalamus had no inhibiting effect on TSH production (12, 20), while thyroxine injections into the adenohypophysis did indeed inhibit thyroid function.

It is likely that a repetition of their experiments using triiodothyronine would show that the local concentration of triiodothyronine in the adenohypophysis is also inhibitory of the production of TSH. However, triiodothyronine might have a similar effect when injected into the hypothalamus, particularly into the paraventricular region, which was not done in the von

Eller study. This region might be of particular interest, since it has shown autoradiographically a high concentration of triiodothyronine.

The concentration of thyroid hormones in the neurohypophysis may be related to the function of that body with respect to water metabolism. Morel and Simon (21) found that the neurohypophyseal thyroxine concentration paralleled its antidiuretic activity. In this connection it may be noted that in hypothyroidism there is an increase above normal (22, 10) and in hyperthyroidism a decrease below normal (23) in the uptake of thyroxine by the neurohypophysis. This would be more compatible with the changes in water metabolism found in these two thyroid states than with mechanisms involved in the regulation of thyroid hormone production. On the other hand, massive doses of ethanol, which block the release of antidiuretic hormone from the neurohypophysis, had no effect on its uptake of thyroxine (10). The interpretation of this finding, however, is complicated by the fact that ethanol also antagonizes the diuretic action of thyroxine (24).

The concentration of triiodothyronine in the gray substance of the brain is of some interest. Its distribution there parallels that of radioactive methionine when used as an indicator of the sites of active protein formation in the brain (25, 26); it would perhaps indicate the site of thyroid hormone action on the growth and myelination of the brain (27). This association of triiodothyronine concentration with sites of anabolic activity has been found to be a striking feature of its distribution in other organs of the body (28). In addition to its role in metabolism and growth, brain triiodothyronine could have a direct action on the functions of the brain (29, 30).

Acknowledgments

The authors wish to acknowledge with much appreciation the assistance, at various stages of the project, of the following persons: M. Buschke, J. Horton, S. Kantounis, L. Kaufman, M. Posner, T. Styles, and S. Symchowicz. The final manuscript was edited by M. Morrow.

REFERENCES

1. Gross, J.: *Brookhaven Symposium in Biology No. 7*, The Thyroid. P. 102. 1955.
2. Manery, J. F. and W. F. Bale: *Am. J. Physiol.* **132**: 215. 1941.
3. Manery, J. F. and L. F. Haege: *Am. J. Physiol.* **134**: 83. 1941.
4. Courrier, R., A. Horeau, M. Marois and F. Morel: *Compt. rend. Soc. Biol.* **143**: 935. 1949.
5. Jensen, J. M. and D. E. Clark: *J. Lab. and Clin. Med.* **38**: 663. 1951.
6. Jentzer, A.: *Acta Endocrinol.* **12**: 264. 1953.
7. Sturm, A. and W. Wernitz: *Acta Neuroveg.* **13**: 50. 1955.
8. Sloviter, H. A. and F. Morel: *Arch. Biochem.* **62**: 217. 1956.
9. Courrier, R., J. Roche, O. Michel, R. Michel and R. M. Andree Colonge: *Compt. rend. Soc. Biol.* **150**: 1125. 1956.
10. Taurog, A. G., G. W. Harris and I. L. Chaikoff: *Endocrinology* **59**: 34, 1956.

11. Gordon, A. H., J. Gross, D. O'Connor and R. Pitt-Rivers: *Nature* **169:** 19. 195?.
12. Euler, C. von and B. Holmgren: *J. Physiol.* **131:** 125. 1956.
13. Gross, J., D. H. Ford, S. Symchowicz and J. H. Horton: *Ciba Foundation Colloquia on Endocrinology* **10:** 182. 1957.
14. Ford, D. H., M. Posner and J. Gross: *Anat. Rec.* **121:** 294. 1955.
15. Gross, J., D. H. Ford and M. Posner: *J. Clin. Endocrinol. & Metab.* **15:** 836. 195?.
16. Tata, J. R., J. E. Rall and R. W. Rawson: *Endocrinology* **60:** 83. 1957.
17. Brown-Grant, K.: *Ciba Foundation Colloquia on Endocrinology* **10:** 97. 1957.
18. Guillemin, R. and B. Rosenberg: *Endocrinology* **57:** 599. 1955.
19. Eartly, H. and C. P. Leblond: *Endocrinology* **54:** 249. 1954.
20. Euler, C. von and B. Holmgren: *J. Physiol.* **131:** 137. 1956.
21. Morel, F. and C. Simon: *Compt. rendu.* **242:** 817. 1956.
22. Harper, E. O. and P. A. Mattis: *Fed. Proc.* **10:** 306. 1951.
23. Harper, E. O., P. A. Mattis and J. W. Boehne: *Fed. Proc.* **11:** 355. 1952.
24. Horger, L. M. and M. X. Zarrow: *Proc. Soc. Exper. Biol. and Med.* **92:** 247. 1956.
25. Cohn, P., M. K. Gaitonde and D. Richter: *J. Physiol.* **126:** 7P. 1954.
26. Flanigan, S., E. R. Gabrieli and P. D. Maclean: *Arch. Neurol. and Psychiat.* **77:** 588. 1957.
27. Eayrs, J. T.: *Nature* **172:** 403. 1953.
28. Ford, H. D., K. Corey and J. Gross: *Endocrinology* (in Press).
29. Timiras, P. S., D. M. Woodbury and S. L. Agarwal: *J. Pharmacol. & Exper. Therap.* **115:** 154. 1955.
30. Timiras, P. S. and D. M. Woodbury: *Endocrinology* **58:** 181. 1956.

THE PRODUCTION OF EXPERIMENTAL EXOPHTHALMOS

ROBERT BRUNISH

Department of Physiological Chemistry, University of California Medical Center, School of Medicine, Los Angeles, California

ABSTRACT

Exophthalmos has been produced in the fish, *Fundulus parvipinnis* by the administration of anterior pituitary preparations. Pepsin digestion does not destroy their effectiveness although it does reduce substantially the thyrotropic activity of such preparations.

EXOPHTHALMOS is a condition with a long and confusing history. It has been associated with thyroid dysfunction, and experimentally it has been produced by anterior pituitary fractions. Thyrotropin (TSH) has caused experimental proptosis under a variety of conditions. Normal guinea pigs (1), thyroidectomized guinea pigs (2), few injections (3), many injections (4), TSH alone (5), or TSH augmented with other hormones (6) have all been employed. These variable requirements have been recently attributed to the contamination of TSH preparations with a specific hormone which causes the changes leading to exophthalmos (7), or to the participation of more than one known hormone; e.g. TSH and corticotropin (8). The results reported herein support the former hypothesis.

In exophthalmos the tissues lying behind the eye show accumulation of water (9) and an increase in mucopolysaccharide content (10). The role of mucopolysaccharide in binding water is well known. The present study records the sequential changes occurring during the onset of exophthalmos to determine if the order of these changes is consistent with the hypothesis that exophthalmos is a reflection of a change in mucopolysaccharide balance. For this purpose protein fractions from the anterior lobe of bovine pituitary glands have been prepared which have the ability to produce exophthalmos in the test animal, *Fundulus parvipinnis*, the common Pacific "minnow." The data obtained are consistent with the stated hypothesis.

MATERIALS AND METHODS

Fundulus parvipinnis were obtained from the waters adjacent to the city of Los Angeles. The fish were maintained in salt water aquaria at 17–18° C, and all experiments were carried out at this temperature. Fish were marked with colored thread sewn through their lower lip or tail at least 24 hours prior to an experiment. The fish, which had been fed minced liver, were fasted for 24 hours prior to an experiment and during its course. The fish weighed from 5 to 10 gm. and, as far as it was possible, the average weight of groups was kept constant.

Received August 28, 1957.

Anterior pituitary fractions were prepared from frozen beef pituitary glands which had been obtained directly from local slaughter houses. Essentially, the method of Ciereszko was employed (11). One hundred grams of dissected anterior lobes were extracted with 500 cc. of 2% sodium chloride at 0–4° C. A Waring blendor replaced the meat grinder of the original procedure. The saline extract was adjusted to pH 4.0. The insoluble protein was removed by centrifugation at 3500 rpm for 20 minutes in an International centrifuge, Model P^{R}–1. An equal volume of acetone was added to the supernatant fluid, and the resulting insoluble fraction was removed with the Sharples Super-Centrifuge at 25,000 rpm. When necessary, the supernatant fluid was clarified further by filtration through a celite pad. Upon the addition of acetone to 75%, the crude active material was obtained as precipitate. This fraction was washed with cold acetone and ether and dried thoroughly. It was then extracted with water, and the resulting solution was lyophilized and stored *in vacuo* over phosphorus pentoxide. This material (Fraction I) had both thryotropic activity (12) and exophthalmic activity as measured in the *Fundulus*. Fraction I was treated with 1% (w/w) pepsin at pH 2.1 at 37° for two hours. Approximately 50% of the starting material was isolated as a non-dialyzable, water soluble mixture of peptides and proteins (Fraction II).

Commercial TSH (Armour's Thytropar) and corticotropin (Armour's ACTHar) were also employed.

Assay of exophthalmic activity followed the method described by Dobyns and Steelman (13). The intercorneal distance of the test fish was measured with calipers to 0.1 mm. prior to and following the test injection. The solution to be assayed was injected intraperitoneally. A 26 gauge needle was used, and the injection was made through the vent and gut wall to prevent leakage which resulted if the injection were made directly through the body wall. The ratio of the intercorneal distance sixteen hours after injection to the pre-injection distance served as a measure of exophthalmos.

Chemical changes induced by the exophthalmic factor were studied. The contents of the fish orbit, except for the globe, optic nerve and conjunctiva were dissected out. Groups of 20–40 fish were employed at varying time intervals following the intraperitoneal injection. The pooled tissue was weighed, dried at 105° C for fifteen hours, and reweighed to determine water content. The hexosamine content of the dried residue was determined, following its chromatographic separation from interfering substances (14), by the colorimetric method of Elson and Morgan as modified by Blix (15).

RESULTS AND DISCUSSION

During the acetone fractionation procedure of Ciereszko the exophthalmic activity was not separated from TSH. As the result of pepsin treatment, Fraction II had greater activity per unit weight than did Fraction I, while Fraction I and Thytropar had similar activities. Dr. John G. Pierce, of this department, assayed Fractions I and II for TSH activity (12) and found that the pepsin treatment substantially reduced the TSH level. Mrs. Jessamine Hilliard, of the Department of Anatomy, determined that this treatment also reduced the level of pepsin-inhibitor, another substance closely associated with TSH (16). In contrast to the results obtained with pepsin, the use of trypsin completely destroyed the exophthalmic activity of Fraction I. Fraction II, at the 5 mg. dose level, gave moderate and reproducible exophthalmos (Fig. 1). At this dosage the intercorneal distance 16 hours following injection was of the order of 106% of the initial distance although occasional values of 110–130% resulted. The dose response curve

fc Fraction II is shown in Figure 2. It might be noted that these fish a pear to be less responsive than the Eastern species, *Fundulus heteroc hus*, both in the degree of exophthalmos and in the length of time re- q ired for maximum proptosis (13). ACTHar, at doses of 5 mg., was in- e ective in producing exophthalmos, nor did it enhance the proptosis pro- d ced by the stimultaneous administration of Thytropar or Fraction II.

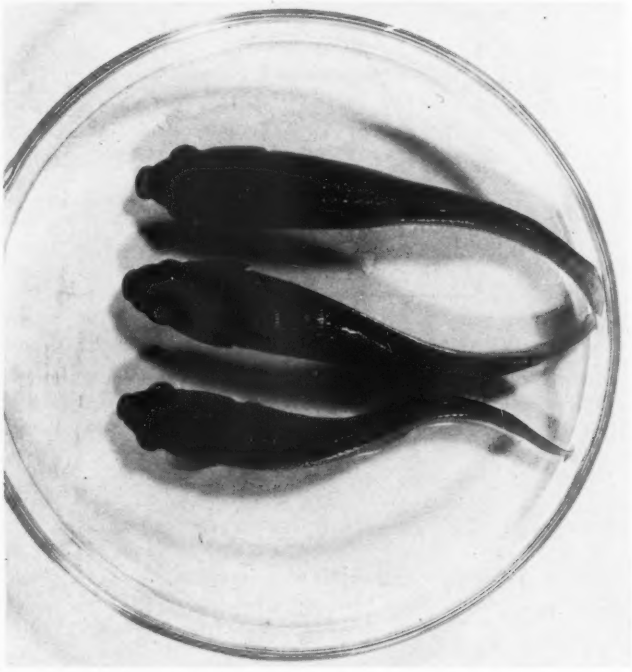


FIG. 1. *Fundulus parvipinnis.* The fish in center received saline injection sixteen hours previously while the other two received 5 mg. of Fraction II.

As a result of its pepsin stability, the exophthalmic activity, as measured in *Fundulus parvipinnis*, would appear to reside in a portion of the TSH molecule or else to exist as a separate entity. There is no evidence to support the theory that intact TSH is the active principal or that it is augmented by ACTH.

The time course of exophthalmos and the concomitant chemical changes associated with it are shown in Figure 3. There is a dip in the values for water content, proptosis and hexosamine concentration at 4–8 hours, followed by a rise in all three.

It might be argued that the water and the hexosamine changes occur independently, and the nature of the injected material is of sufficient complexity for this view. Pepsin treatment has reduced the levels of two addi-

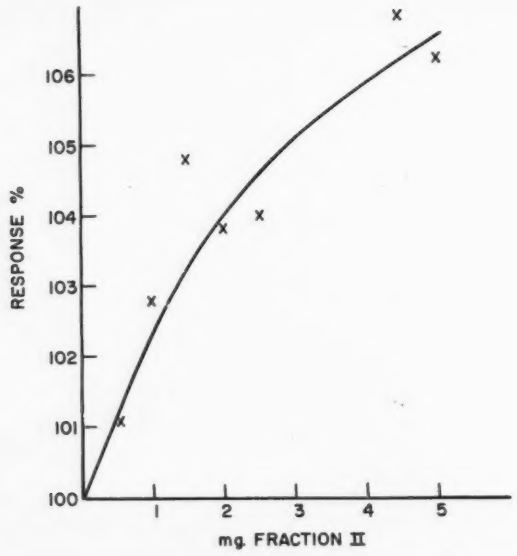


FIG. 2. Dose-response curve. The ratios of the intercorneal distances obtained sixteen hours following injection to their preinjection values are plotted against the weight of Fraction II administered. Each point represents the average value obtained from 5-8 fish.

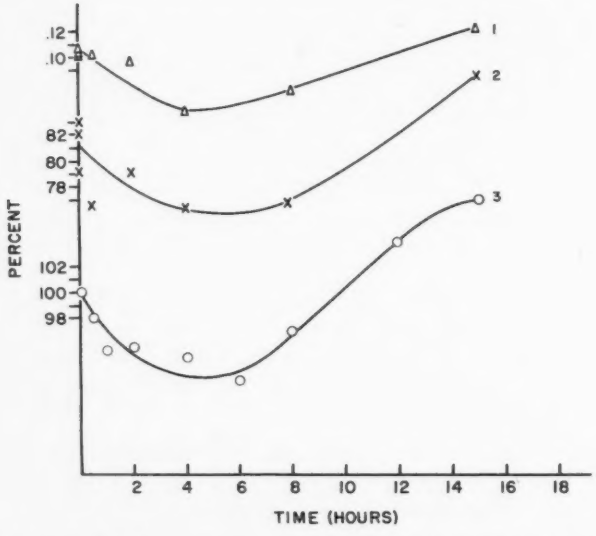


FIG. 3. The sequential changes occurring during the incipient stages of exophthalmos. Five mg. of Fraction II were injected intraperitoneally. Curve 1 represents the per cent hexosamine, on a dry-weight basis in the orbital tissue; 2, the per cent water; 3, the intercorneal distance relative to the preinjection value.

tional substances which previously had been associated with anterior pituitary preparations producing exophthalmos, namely TSH and the trypsin-inhibitor. Nonetheless until it is shown that the factors governing the water change and the hexosamine response can be separated chemically or physically, there is no reason to reject the hypothesis that the mucopolysaccharide change governs the water balance and that this in turn regulates the degree of proptosis. The data obtained are consistent with this hypothesis.

Acknowledgments

This investigation was supported in part by the Estelle Doheny Eye Foundation and by the National Council to Combat Blindness, Inc. Grant #182-C.

REFERENCES

1. Jefferies, W. McK.: *J. Clin. Endocrinol.* **9**: 927. 1949.
2. Smelser, G. K.: *Am. J. Ophthal.* **20**: 1189. 1933.
3. Ludwig, A. W.: *Proc. Soc. Exp. Biol. and Med.* **85**: 424. 1954.
4. Nuzum, J. W.: *Surgery* **33**: 550. 1953.
5. Smelser, G. K. and V. Ozanics: *Am. J. Ophthal.* **34**: No. 5, Pt. II, 87. 1951.
6. Smelser, G. K. and V. Ozanics: *Am. J. Opthal.* **39**: No. 2, Pt. II, 147. 1955.
7. Jefferies, W. McK.: *Arch. Ophthal.* **56**: 671. 1956.
8. Aterman, K.: *Acta Endocrinol.* **15**: Suppl. 20. 1954.
9. Smelser, G. K.: *Am. J. Physiol.* **140**: 308. 1943.
10. Ludwig, A. W., N. F. Boas and L. J. Soffer: *Proc. Soc. Exp. Biol. and Med.* **73**: 137. 1950.
11. Ciereszko, L.: *J. Biol. Chem.* **160**: 585. 1945.
12. Greenspan, F. S., J. P. Kriss, L. E. Moses and W. Lew: *Endocrinology* **58**: 767. 1956.
13. Dobyns, B. M. and S. L. Steelman: *Endocrinology* **52**: 705. 1953.
14. Boas, N. F.: *J. Biol. Chem.* **204**: 553. 1953.
15. Blix, G.: *Acta Chem. Scand.* **2**: 467. 1948.
16. Hilliard, J. and P. H. West: *Endocrinology* **68**: 797. 1957.

THE EFFECT OF VENTRAL HYPOTHALAMIC LESIONS ON SODIUM AND POTASSIUM METABOLISM IN THE DOG[1,2]

WILLIAM J. R. DAILY AND WILLIAM F. GANONG

From the Department of Physiology, School of Medicine, University of California, Berkeley, California

ABSTRACT

The urinary excretion and plasma levels of sodium and potassium in 18 dogs with ventral hypothalamic lesions have been compared to those found in normal, hypophysectomized and adrenalectomized animals on a daily diet containing 40 m.eq. of sodium and 8 m.eq. of potassium, on a diet containing 0.5 m.eq. of sodium and 50 m.eq. of potassium, and after the intravenous administration of a salt load equivalent to 5.13 m.eq. per kilogram of body weight.

The ventral hypothalamic lesions produced diabetes insipidus, gonadal atrophy and/or inhibition of hydroxycorticoid secretion in response to surgical trauma in a large number of the animals studied. However, none of the animals showed abnormal sodium and potassium metabolism and all withstood salt restriction and handled a salt load in a normal fashion. Hypophysectomized dogs also responded normally, but adrenalectomized dogs, maintained before and during the diet studies on hydrocortisone alone, became hyponatremic and died when subjected to salt restriction.

DESTRUCTION of the anterior end of the median eminence of the hypothalamus is known to prevent the increased secretion of ACTH and adrenal hydroxycorticoids that normally follows psychic or physical trauma in the dog (1, 2, 3). Whether or not this deficiency in glucocorticoid secretion is associated with changes in the metabolism of sodium and potassium apparently has not been determined, and the ability of such animals to withstand the "metabolic stress" of sodium deprivation has not been studied. Accordingly, the present experiments were undertaken to investigate serum levels and urinary excretion of sodium and potassium in dogs with lesions in the median eminence and other parts of the ventral hypothalamus, and to observe the changes in these parameters that follow salt restriction and salt loading.

Received September 9, 1957.

[1] These studies were supported by a grant-in-aid from the Commonwealth Fund. The data were submitted by the senior author in partial fulfillment of the requirements for the M.A. degree in Physiology at the University of California.

[2] Reported in preliminary form in a paper read at the 38th Annual Meeting of the Endocrine Society, New York, May, 1957.

MATERIALS AND METHODS

Forty-one male mongrel dogs weighing 6.8 to 23.5 kg. were studied. 15 of these animals were normal, control dogs. 18 had lesions in the ventral hypothalamus. In addition, five hypophysectomized and three adrenalectomized dogs were studied. When not on measured diet studies, the dogs were routinely fed a commercial dog meal (Square Deal Dog Food, Hygienic Dog Food Co., Berkeley, California).

Electrolyte metabolism in these animals was studied in the following situations:

1. While consuming a diet containing 40 m.eq. of sodium and eight m.eq. of potassium per day (the "40 m. eq. sodium diet"). The animals were placed on this regime for two days, while two 24-hour urine specimens and blood samples at the beginning and end of the 48-hour period were obtained.

2. While consuming a diet containing 0.5 m. eq. of sodium and 50 m. eq. of potassium per day (the "0.5 m.eq. sodium diet"). The dogs were placed on this regime for a three-day period. Three 24-hour urine specimens and blood samples at the beginning and end of the period were collected.

3. Following salt loading. All dogs were given an intravenous dose of 15% sodium chloride, equivalent to 5.13 m.eq. per kg. body weight. Urine samples were obtained three hours thereafter. Similarly, blood samples were obtained before and three hours after the injection. This procedure was routinely carried out after two days on the 40 m.eq. sodium diet. After the three-hour urine and blood specimens had been obtained, the dogs were fed the daily amount of the 40 m.eq. sodium diet, and the study terminated with another urine and blood sample at the end of 24 hours.

In several of the normal dogs and a number of the dogs with lesions, the 40 m.eq. sodium and the 0.5 m.eq. sodium diet were each carried out for periods of as long as five days.

All urine samples were obtained in metabolic cages. The urine remaining in the bladder at the end of each collection period was removed by catheterization, using a number six French woven ureteral catheter. The bladder was washed with 20–30 ml. of distilled water and this amount added to the total specimen. The volume of the specimen was measured and an aliquot of the mixed total urine sample refrigerated for analysis. Blood samples were allowed to clot, then centrifuged promptly and the serum stored in the refrigerator. In addition, an aliquot of each batch of diet was routinely saved in the refrigerator for subsequent analysis.

The diets were prepared with Lonalac (Mead Johnson and Company). 42.6 gm. of this material made up to 300 ml. with isotonic saline solution provided a dog with an intake of 40 m.eq. of sodium and 8 m.eq. of potassium, while the same amount of Lonalac made up to 300 ml. with isotonic potassium chloride supplied 0.5 m.eq. of sodium and 50 m.eq. of potassium. Although this amount of Lonalac supplied approximately 10 gm. of protein daily, the fact that all normal animals lost weight slowly on this diet indicates that it was deficient in total calories. However, all dogs did well on it except hypophysectomized animals. In order to prevent hypoglycemic complications in these animals, 100 gm. of dextrose per day was added to the daily ration. Routinely, the dietary mixture was made up in bulk lots adequate to supply two to five animals throughout a one week experimental period. The fluid mixture was kept refrigerated, and under these conditions, the mixture remained usable throughout the week. Any diet remaining in the dogs' pans at the end of twenty four hours was measured and subtracted from the daily intake. During these studies, the dogs were allowed water *ad lib.* This water was analyzed for sodium and potassium content and found to contain negligible amounts of these ions.

The urine, diet and serum samples were subsequently appropriately diluted and analyzed for potassium and sodium content, using a Baird flame photometer with lithium as the internal standard.

The electrolytic lesions were placed stereotaxically in the hypothalamus by the technique of Hume and Ganong (4). There were no deaths in this series and all dogs did well. The studies of electrolyte excretion were performed three to seven weeks postoperatively. In addition to the determinations already mentioned, body temperatures were checked at least twice with a thermister device. At least three 24-hour urines were checked for the presence of sugar. Body weights were determined at the start of and end of each dietary study.

Hypophysectomies were performed trans-bucally and the dogs studied three weeks later. Adrenalectomies were performed in two stages through flank incisions.

When the electrolyte metabolism studies were completed, the dogs were subjected to adrenal vein cannulation under pentothal-induced ether anesthesia by the method of Hume and Nelson (5). A timed adrenal blood sample was obtained and the animal then given intravenously one I.U. of ACTH. Four minutes later, another timed adrenal venous sample was obtained. Samples were collected in chilled, heparinized graduated centrifuge tubes and the plasma separated immediately. The samples thus obtained were stored at 0° C. The 17-hydroxycorticoid content of the plasma was subsequently determined by the method of Silber and Porter (6). The adrenal hydroxycorticoid output was then calculated in terms of gamma per minute.

Following adrenal vein cannulation, the carotid arteries were freed and cannulated. The head was perfused with isotonic saline followed by 10% neutral formalin. The gonads and adrenal glands were removed and weighed, the left and right adrenals being weighed separately. One of the gonads, the left adrenal and a piece of the right kidney were fixed in formalin for subsequent histological examination after staining with hematoxylin and eosin. The hypothalamus and pituitary were removed as a unit by sawing through the head. The block of tissue thus obtained was serially sectioned at 16 μ and and every tenth section was stained with Bullard's modification of Ehrlich's hematoxylin and eosin stain. Every fifth slide was traced on paper, using a projector which magnified 10 times. From these drawings, a reconstruction of the lesion on a drawing of a sagittal section of the hypothalamus was made for each dog.

RESULTS

It should be emphasized that because stool electrolytes were not determined, these studies are not true balance studies. However, none of the dogs developed diarrhea, and sodium excretion in the urine was approximately equal to dietary intake on the 40 m.eq. sodium diet. Most of the animals showed a potassium excretion in excess of intake on this diet, probably reflecting the caloric inadequacy of the diet. These results are summarized in Table 1.

When the normal animals were put on the 0.5 m.eq. sodium diet, sodium excretion exceeded intake by 12.5 ± 1.93[3] m.eq. on the first day, 5.6 ± 0.7 on the second and 4.6 ± 0.6 on the third. In dogs carried on this diet for five days, sodium excretion did not decline further, so sodium conservation was apparently maximal in these dogs by the third day of salt restriction. On this regime, normal dogs excreted an amount of potassium approximating dietary intake, as shown in Table 1.

The mean serum sodium level of the normal dogs on the second day of the 40 m.eq. sodium diet was 142.9 m.eq./liter. At the start of the low salt

[3] Standard error of the mean.

diet, it was 144.2 m.eq./liter, and after three days of salt restriction, it was 144 m.eq./liter. The corresponding serum potassium values were 4.5, 4.7 and 4.9 m.eq./liter.

The previously reported fact that hypophysectomy has little effect on sodium and potassium metabolism (7, 8) was confirmed. Two of the five hypophysectomized dogs developed severe hypoglycemia. In the remaining three animals, this was prevented by adding 100 gm. of dextrose to the balance diet daily, but one of these dogs proved at autopsy to have severe nephritis. Results in all animals were similar, but only the data collected on the two otherwise normal dogs have been included. As shown in Table 1, these dogs showed an average excess of sodium intake over excretion of 1.2 and 2.5 m.eq. on day one and two of the 40 m.eq. sodium diet, and potassium excretion in excess of intake by 12.9 and 4.1 m.eq. respectively. On the low sodium, high potassium diet, their values for intake minus urinary excretion of sodium and potassium were practically identical to the mean values for the normal dogs. Serum sodium and potassium levels were normal throughout.

All but four of the 18 animals with ventral hypothalamic lesions showed one or more of a variety of endocrine abnormalities. Eight of these had adrenal venous hydroxycorticoid outputs in response to surgical trauma of less than 3.7 gamma per minute. This value compares to a mean normal response in this laboratory (15 dogs) of 6.9 ± 0.5 gamma per minute. Six dogs had diabetes insipidus (urinary output of 800 ml. or more on two consecutive days) and nine dogs had gonadal atrophy (gonadal weight less than 1 gm. per kg. body weight).

The individual lesions in the dogs with depressed hydroxycorticoid outputs destroyed bilaterally various portions of the area between the optic chiasm and the anterior edge of the mammillary body, extending dorsally in a few cases as much as three mm. above the median eminence and laterally two mm. from the midline. However, all these animals had in common a midline area of destruction in the anterior end of the median eminence (Fig. 1). Gonadal atrophy occurred in dogs with lesions of variable extent, some extending dorsally to the paraventricular nuclei, but it was never present unless a small portion of the median eminence in the region of the infundibulum was destroyed. This area common to all dogs with gonadal atrophy is shown in Figure 2. In one dog with moderately severe diabetes insipidus, the area destroyed was limited to the area immediately ventral to the paraventricular nuclei bilaterally, without severe damage to the supraoptic region. In the other five dogs with this syndrome, the lesions were in or near the posterior end of the supraoptic nuclei. Some extended well into the optic chiasm, and a few also destroyed areas well posterior to this region, but these five animals all had a common area of destruction (Fig. 2) in and just above the postero-dorsal aspect of the optic chiasm and extending laterally for a distance of two mm. on either side. In view

of McCann and Brobeck's hypothesis that vasopressin is the hypothalamic neurohumor which stimulates ACTH secretion (10), it is interesting to note that two of the dogs with severe diabetes insipidus had normal hydroxycorticoid outputs in response to surgical trauma. The four animals without polyuria or gonadal or adrenal abnormalities all had lesions in and above the optic chiasm.

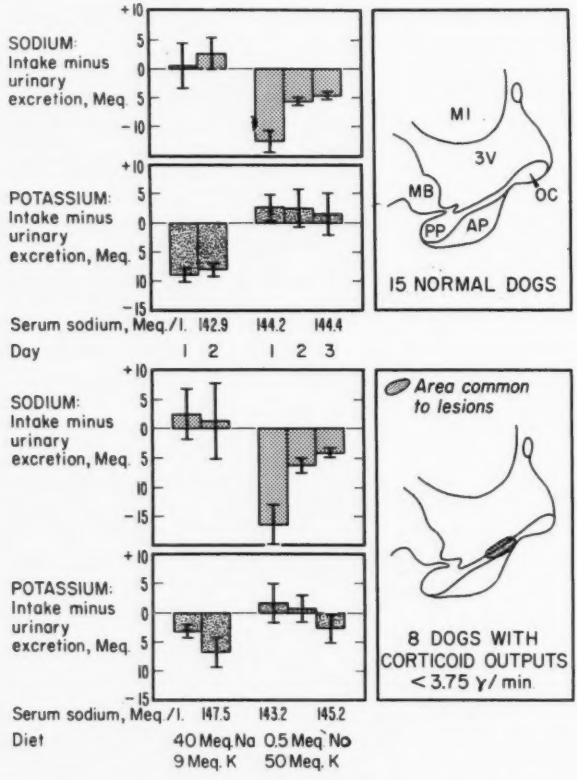

SODIUM: Intake minus urinary excretion, Meq.
POTASSIUM: Intake minus urinary excretion, Meq.
Serum sodium, Meq./l. 142.9 144.2 144.4
Day 1 2 1 2 3
MI
3V
MB
OC
PP
AP
15 NORMAL DOGS
Area common to lesions
8 DOGS WITH CORTICOID OUTPUTS < 3.75 γ/min
Serum sodium, Meq./l. 147.5 143.2 145.2
Diet 40 Meq. Na 9 Meq. K 0.5 Meq. Na 50 Meq. K

FIG. 1. Sodium and potassium metabolism in normal dogs and dogs with hypothalamic lesions and depressed 17-hydroxycorticoid outputs on a diet containing 40 m.eq. of sodium (first two bars) and 0.5 m.eq. of sodium (last three bars). The shaded bars represent mean values and the small bars represent ± the standard error of the mean. The diagram on the right represents, in each case, a sagittal section of the hypothalamus. MI, massa intermedia; MB, mammillary body; PP, posterior pituitary; OC, optic chiasm; 3V, third ventricle; AP, anterior pituitary.

The urinary excretion of sodium and potassium by animals with depressed hydroxycorticoid outputs is compared to that of normal animals and other dogs with hypothalamic lesions on the various dietary regimes in Table 1. On the 40 m.eq. sodium diet, dietary intake of sodium was

slightly greater than urinary excretion on both days of the study, the values being similar to those found in the normal animals. On the low sodium diet, the differences between sodium intake and urinary output were -16.5 ± 3.3 m.eq. on day one, -6.3 ± 1.3 m.eq. on day two and -4.2 ± 0.7

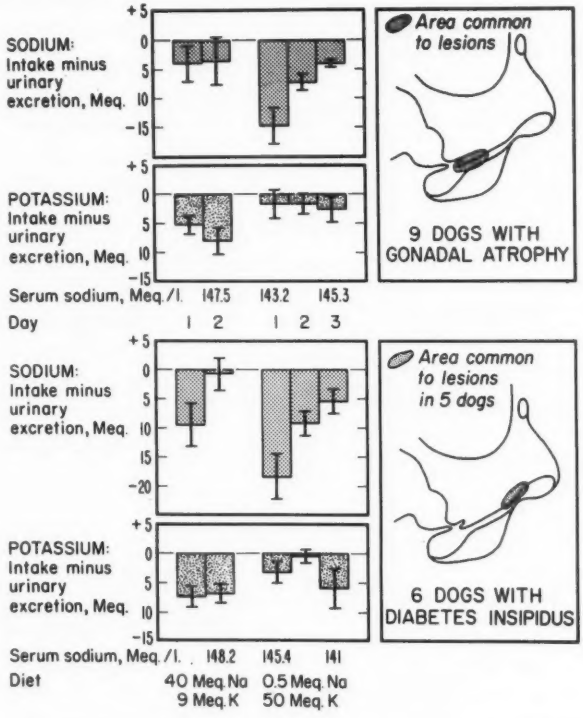

SODIUM: Intake minus urinary excretion, Meq.
POTASSIUM: Intake minus urinary excretion, Meq.
Serum sodium, Meq./l. 147.5 143.2 145.3
Day 1 2 1 2 3
Area common to lesions
9 DOGS WITH GONADAL ATROPHY
SODIUM: Intake minus urinary excretion, Meq.
POTASSIUM: Intake minus urinary excretion, Meq.
Serum sodium, Meq./l. 148.2 145.4 141
Diet 40 Meq. Na 9 Meq. K 0.5 Meq. Na 50 Meq. K
Area common to lesions in 5 dogs
6 DOGS WITH DIABETES INSIPIDUS

FIG. 2. Sodium and potassium metabolism in dogs with gonadal atrophy and those with diabetes insipidus.

m.eq. on day three of the diet. Mean serum sodium values were 147.5 m.eq./L on the second day of the 40 m.eq. sodium diet, 143.2 m.eq./L at the start of salt restriction and 145.2 m.eq./L at the end. None of the individual dogs in this group showed significantly abnormal serum sodium or potassium levels at any time during the experiments. Thus, although hydroxycorticoid output after surgical trauma was abnormally low in these animals, they were able to withstand the "metabolic stress" of a low sodium, high potassium diet in a perfectly normal fashion.

The results of the experiments in the dogs with gonadal atrophy and diabetes insipidus are summarized in Figure 2. Average sodium excretion in the former dogs was slightly greater than their sodium intake, but these values are not significantly different from those found in normal animals. Similarly, all animals showed a normal pattern of sodium conservation on

the 0.5 m.eq. diet and average serum sodium and potassium values were not essentially different from the normal. In the dogs with diabetes insipidus, urinary excretion of sodium on the first day of the 40 m.eq. sodium diet exceeded dietary intake by 9.6 m.eq. However, on the second day, excretion equaled intake, and the value for the first day is not significantly different statistically from the mean value for the first day for normal animals. The response to salt restriction and the serum sodium and potassium levels were normal.

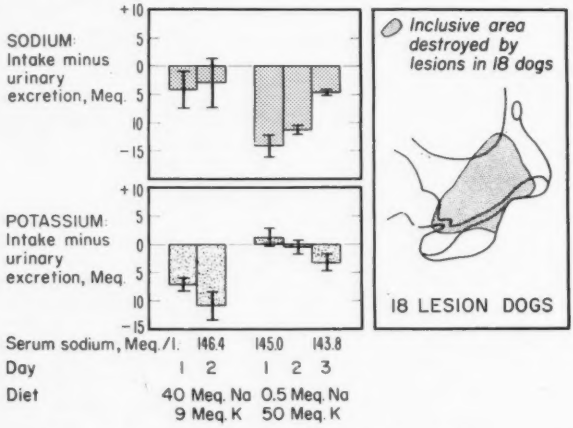


FIG. 3. Sodium and potassium metabolism in all dogs with hypothalamic lesions. Note that shaded area on the diagram of the sagittal section of the hypothalamus represents the composite area destroyed by all the lesions, rather than the area common to them.

Figure 3 summarizes the data from all eighteen days with hypothalamic lesions taken as a group. This includes the four dogs which showed no definite endocrine abnormalities as well as the dogs with diabetes insipidus, gonadal atrophy, and deficient hydroxycorticoid secretion in response to trauma. Again, the values for dietary intake minus urinary excretion of sodium and potassium are not significantly different from those observed in the normal animals on either the 40 m.eq. sodium diet or the 0.5 m.eq. diet regime, and the serum sodium and potassium levels are similar to those of the normal animals. The shaded area in the chart in Figure 3 represents the inclusive total area destroyed by lesions in the eighteen dogs. It appears fair to conclude, therefore, that the area represented by these lesions is not concerned with the maintenance of sodium and potassium balance on a normal sodium intake, nor does it contain centers essential to the mechanisms by which the dog responds to salt deprivation.

These results are in contrast to those obtained in the adrenalectomized dogs. These three animals were maintained without mineralo-corticoid

therapy on the stock diet, which contained approximately 40 m. eq. of sodium daily, and they received a daily dose of 5 mg. of hydrocortisone (free alcohol) intramuscularly for 11 days postoperatively. At that time, severe hyponatremia was present, the mean serum sodium being 122.7 m.eq./L and the serum potassium 6.1 m.eq./L. Placed on the measured 40 m.eq. sodium diet at that point, they excreted only a fraction of their daily intake in the urine (Table 1). Despite this retention, their serum sodium levels fell slightly, to a mean value on the second day of 120.7 m.eq./L. When placed on the low sodium, high potassium diet, all three animals died within 24 hours.

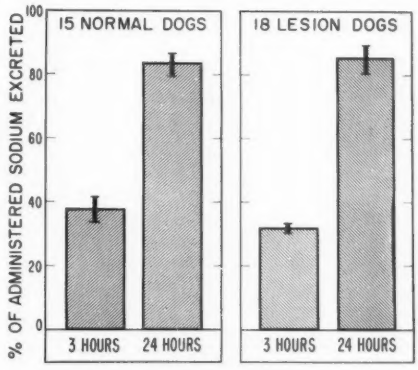


FIG. 4. Per cent excretion of an intravenous salt load three hours after injection and of intravenous plus dietary sodium in 24 hours by normal dogs and all dogs with hypothalamic lesions. The shaded bars represent mean values and the small bars ± the standard error of the mean.

The adrenalectomized animals also excreted an average of only 9.4% of the administered salt load in three hours, and 53.7% in 24 hours. This compares to a mean three hour excretion in the normal dogs of 37.4 ±3.8% and a 24-hour value of 83.1 ±3.8%. The hypophysectomized dogs, on the other hand, excreted 34.1% of the salt load in three hours and 94.5% of their intake in 24 hours. The majority of the animals with lesions handled a salt load like the normal and the hypophysectomized dogs (Fig. 4 and 5). Thus, the mean three and 24-hour per cent excretion values for the dogs with gonadal atrophy were 32.6 ±4.4% and 90.6 ±2.6%, those for the dogs with diabetes insipidus 35.2 ±4.8% and 87.8 ±4.1%. The only possibly abnormal group were the dogs with depressed hydroxycorticoid output. They excreted 27.9 ±5.3% of the load in three hours and 79.1 ±3.2% in 24 hours. This is reminiscent of the deficient response to salt loading in adrenalectomized animals, but the differences between the mean values of this group of dogs with lesions and the normals is not significant statistically.

The histology and weight of the adrenals of all of the dogs with lesions were not significantly different from those of the normal dogs. Atrophy of the zona glomerulosa was not observed. Atrophic changes in the seminiferous tubules and the Leydig cells were found in the testes of animals with

TABLE 1. SODIUM AND POTASSIUM EXCRETION OF NORMAL, HYPOPHYSECTOMIZED AND ADRENALECTOMIZED DOGS, AND DOGS WITH HYPOTHALAMIC LESIONS

Data is presented for the animals with lesions as a group, and also for the animals with lesions showing each of three endocrine abnormalities. Note that animals showing more than one endocrine abnormality are included in more than one of these latter subdivisions. Values are means ± standard error of the mean.

Preparation	No. of dogs	Dietary intake minus urinary excretion (meq.)									
		Diet: 40 m.eq. Na, 8 m.eq. K				Diet: 0.5 m.eq. Na, 50 m.eq. K					
		Sodium		Potassium		Sodium			Potassium		
		Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Normal	15	+0.5±3.9	+2.0±2.7	−8.8±1.2	−7.9±1.2	−12.5±1.9	−5.6±0.7	−4.6±0.6	+2.7±2.2	+2.5±3.2	+1.4±3.6
Adrenalectomized	3	+15.9	+26.4	−6.9	−3.6	—	—	—	—	—	—
Hypophysectomized	2	+1.2	+2.5	−12.9	−4.1	−11.5	−5.8	−5.8	−2.6	−1.6	+2.0
All lesion dogs	18	−4.1±3.3	−2.9±4.4	−7.0±1.2	−10.8±2.5	−14.1±1.9	−11.3±0.8	−4.6±0.5	+1.3±1.7	−0.5±1.2	−3.2±1.5
Dogs with lesions and:											
A. corticoid output<3.7 μg./min.	8	+2.4±4.3	+1.2±6.4	−3.2±1.2	−6.8±2.6	−16.5±3.3	−6.3±1.3	−4.2±0.7	+1.3±1.7	−0.5±1.2	−3.2±1.5
B. gonadal atrophy	9	−3.9±3.1	−3.5±4.1	−5.3±1.6	−8.1±2.2	−14.7±3.1	−7.2±1.4	−4.0±0.5	−1.7±2.4	1.7 ±1.8	−2.6±2.2
C. diabetes insipidus	6	−9.6±3.7	−0.8±2.7	−7.9±1.8	−6.9±1.6	−18.5±4.0	−9.3±2.2	−5.5±2.1	−3.3±1.8	−0.6±1.2	−6.0±3.4

gonads weighing less than 1 gm./kg. body weight. Except for the nephritis in one of the hypophysectomized dogs, no definite renal abnormalities were seen.

The body weight changes of the dogs with lesions during the measured diet studies were comparable to those in the normal and hypophysectomized dogs. None of the dogs developed hyperthermia, and glycosuria was not found.

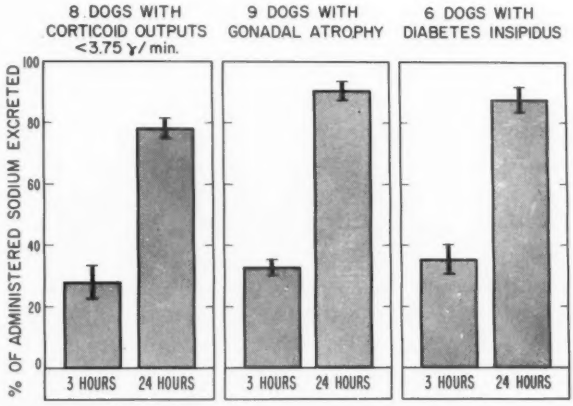


FIG. 5. Per cent excretion of administered sodium at three and 24 hours by the dogs with hypothalamic lesions associated with depressed corticoid outputs, with gonadal atrophy, and with diabetes insipidus.

DISCUSSION

The presence of normal sodium and potassium metabolism in hypophysectomized dogs, their ability to conserve salt on a salt-restricted diet, and their normal handling of an acutely administrered salt load have all been reported previously (7, 8, 9), as has the reduced ability of the adrenalectomized dog to excrete a salt load, even after cortisone therapy (7, 9, 11). The finding, in our adrenalectomized dogs, of sodium retention on the 40 m.eq. sodium diet is interesting, but it must be remembered that these three animals were being and had been maintained for the previous 11 days on a low dose of hydrocortisone without any mineralo-corticoid therapy. Consequently, they were severely hyponatremic at the time the study was performed, and presumably had depressed renal function, both factors probably depressing the filtered sodium load to a very low level (7).

The occurrence of hypernatremia in patients with cerebral disease has been the subject of numerous reports in the clinical literature and several recent reviews (12, 13). Some authors have attempted to inplicate the hypothalamus in the genesis of this hypernatremia (14). Rarer, but better

documented are the cases of so called "cerebral salt-wasting" in association with a variety of cerebral lesions (15).

In spite of this extensive clinical literature, there is little experimental evidence linking the hypothalamus in particular and the brain in general to the control of electrolyte metabolism in animals. Lewey and Gassman (16) placed electrodes in the "paroptic" region of the hypothalamus unilaterally, passed coagulating currents down these electrodes, and followed chloride levels in blood and urine for several hours. They found that this maneuver produced hyperchloremia and hypochloruria. Stevenson, Welt and Orloff (17) compared the serum sodium levels in rats with hypothalamic obesity due to lesions of the ventromedial nuclei with those found in normal animals, and found a slight hypernatremia in the animals with lesions which they attributed to chronic, mild hypodypsia.

We were interested in approaching this problem in animals with stereotaxically produced chronic lesions. The use of chronic animals avoids the difficulty of interpreting whether results such as those of Lewey and Gassman are due to stimulation, to destruction of structures in the brain, or to the trauma coincident to the experimental procedure. We were also particularly interested in investigating the possible role of the ventral hypothalamus because of the evidence that the median eminence is involved in the control of hydroxycorticoid secretion (1, 2, 3). The results obtained clearly indicate that the "unstressed" dog with destruction of the ventral hypothalamus shows no abnormality of sodium and potassium metabolism while consuming a diet containing 40 m. eq. of sodium daily. This is true whether or not such lesions produce inhibition of hydroxycorticoid release, gonadal atrophy and/or diabetes insipidus. Furthermore, such animals are capable of withstanding a low sodium, high potassium diet for periods of three days or longer without ill effects or appreciable fall in the serum sodium level. Finally, the response to salt loading in these animals is normal.

Recently, Rauschkolb and Farrell (18, 19) have advanced the hypothesis that there is a center in the diencephalon which controls, through a humoral mechanism, the secretion of aldosterone. This conclusion was based on the observation that decapitation and decerebration of the dog acutely lowered the amount of aldosterone secreted in the adrenal vein.

We have tried to determine if the techniques used in our experiments would detect a chronically depressed secretion of aldosterone by studying adrenalectomized dogs maintained on basal gluco-corticoid therapy without any mineralo-corticoid replacement. The three dogs so studied all developed severe hyponatremia on this regime, and they all died within 24 hours when placed on the low sodium, high potassium diet. It appears reasonable to conclude, therefore, that if any severe depression in aldosterone output had occurred in the animals with hypothalamic lesions

during the particular experimental situations studied, hyponatremia and inability to withstand the low sodium, high potassium diet would have been evident.

It should be emphasized, however, that the results in the present experiments do not necessarily disagree with those of Rauschkolb and Farrell (18, 19). In the first place, these investigators have not localized their "aldosterone area" to any specific portion of the diencephalon. Figure 3 shows that large areas of the dorsal hypothalamus and rostral midbrain have been left untouched in our studies. Furthermore, Rauschkolb and Farrell measured the actual output of aldosterone in the adrenal vein after the acute trauma of inserting an adrenal venous cannula. Llaurado (20) has presented evidence that humans respond to surgical trauma with increased aldosterone output. It is possible, therefore, that diencephalic centers mediate the release of aldosterone in response to surgical trauma while not effecting the changes in its secretion induced by changes in electrolyte intake. In order to investigate this possibility, we have measured aldosterone output in the adrenal vein blood of a number of the dogs in the present series. These studies, conducted in collaboration with Dr. Amos Lieberman, Dr. Patrick Mulrow and Dr. John Leutscher of the Stanford University School of Medicine, are incomplete as yet. However, preliminary results suggest that lesions which inhibit the release of hydroxycorticoids also partially inhibit the release of aldosterone in animals traumatized by the insertion of an adrenal venous cannula, while not affecting resting sodium and potassium metabolism or the ability to withstand salt restriction.

Acknowledgments

The technical assistance of Miss Angela Boryczka is gratefully acknowledged. We wish to thank Dr. Robert C. Little, of Mead Johnson and Company for the Lonalac, Dr. Sanford L. Steelman of Armour Laboratories for the ACTH, and Dr. Elmer Alpert of Merck, Sharpe and Dohme for part of the hydrocortisone used in these experiments.

REFERENCES

1. Ganong, W. F., N. I. Gold and D. M. Hume: *Fed Proc.* **14**: 54. 1955.
2. Hume, D. M.: *Ann. Surg.* **138**: 548. 1953.
3. Hume, D. M. and D. H. Nelson: *J. Clin. Endocrinol. and Metab.*, **15**: 839. 1955.
4. Hume, D. M. and W. F. Ganong: *Electroencephalog. and Clin. Neurophysiol.* **8**: 136. 1956.
5. Hume, D. M. and D. H. Nelson: Surgical Forum, Am. College of Surgeons, 1954, p. 568, W. B. Saunders and Co., 1955.
6. Silber, R. H. and C. C. Porter: *J. Biol. Chem.* **210**: 923. 1954.
7. Selkurt, E. E.: *Physiol. Rev.* **34**: 287. 1954.
8. Earle, D. P., Jr., R. C. deBodo, E. L. Schwarts, S. J. Farber, M. Kurtz, and J. Greenberg: *Proc. Soc. Exper. Bio. and Med.* **76**: 60-8. 1951.
9. Martin, H. F. and H. L. White: *Am. J. Physiol.* **170**: 532. 1952.

10. McCann, S. M. and J. R. Brobeck: *Proc. Soc. Exper. Biol. and Med.* **87**: 318. 1954.
11. Gross, F. and R. Meier: *Schweiz. Med. Wschr.* **81**: 1013. 1951.
12. Schoolman, H. M., A. Dubin and W. S. Hoffman: *Arch. Int. Med.* **95**: 15. 1955.
13. Anthonisen, P., T. Hilden and A. C. Thomsen: *Nord. Med.* **53**: 713. 1955.
14. Sweet, W. H., G. C. Cotzias, J. Seed and P. Yakovlev: *Proc. Assoc. Res. Nerv. and Mental Dis.* **27**: 795. 1958.
15. Peters, J. P., L. G. Welt, E. A. H. Sims, J. Orloff and J. Needham: *Tr. A. Am. Physicians* **63**: 57. 1950.
16. Lewey, F. H. and F. K. Gassman: *Am. J. Physiol.* **112**: 504. 1935.
17. Stevenson, J. A. F., L. G. Welt and J. Orloff: *Am. J. Physiol.* **161**: 35. 1950.
18. Rauschkolb, E. W. and G. L. Farrell: *J. Clin. Endocrinol. and Metab.* **16**: 915. 1956.
19. Rauschkolb, E. W. and G. L. Farrell: *Endocrinology* **59**: 526. 1956.
20. Llaurado, J. G.: *Lancet* **1**: 1295. 1955.

METABOLISM OF HYDROCORTISONE DURING INFLAMMATION[1]

THOMAS F. DOUGHERTY, HERBERT E. BROWN AND DAVID L. BERLINER

Department of Anatomy, University of Utah College of Medicine, Salt Lake City, Utah

ABSTRACT

The metabolism of cortisol 4-C^{14} was studied in mice subjected to an inflaming stimulus. There was a non-specific concentration of radioactivity in the inflamed connective tissue, demonstrated by the fact that although the amount of hormone is greater on the inflamed side, the rate of increase and decrease of hormone concentration is the same in both inflamed and non-inflamed connective tissue. Further, the tissue concentration of tetrahydrocortisol glucuronide which is the transformation product of cortisol by the liver, reflects the concentration in blood. 50% of the cortisol is inactivated in 40 to 60 minutes, but the anti-inflammatory effect is maintained for a much longer period of time. Therefore, it is apparent that the hormone is concentrated in the inflamed tissue in a non-specific manner, exerts its anti-inflammatory effect, and is excreted. The transformation of cortisol to tetrahydrocortisol and to tetrahydrocortisol glucuronide occurs in mice in the same manner as in humans.

INTRODUCTION

IT IS now well established that 4 pregnene 11,17,21 triol, 3,20 dione (cortisol or hydrocortisone) has a unique and highly potent anti-inflammatory action. The results of numerous experiments indicate that cortisol exerts its antiphlogistic effect within an inflamed volume of tissue (1, 2). It acts apparently by inhibiting the local cell damage and preventing influx of nonautochthonous cells into injured tissue (3). The relatively greater clinical effect of cortisol injected directly into arthritic joints has also suggested an *in situ* activity (4). The question has been raised as to whether cortisol itself or a metabolic product was the active anti-inflammatory agent (2). In the experiments reported here the metabolism of cortisol 4-C^{14} was studied to determine 1) whether qualitative or quantitative differences in distribution of the radioactive substance were present in normal and inflamed connective tissue and in the blood; 2) if differences in metabolic products could be observed in these tissues; and 3) the relative differences in the amounts of hormone present in normal and inflamed connective tissue.

Received September 12, 1957.

[1] This work was supported by grants from the U.S.P.H.S. (No. 34–5219—Leukemogenesis) and from the Department of Army (No. DA—99-007-MD-130).

METHODS

Ten female mice of a C-57 black sub-strain, 12 weeks of age, were used in these experiments. The animals were adrenalectomized 4 days before hormone injection. A topical inflammation was produced 6 hours prior to hormone injection by administering 0.2 ml of a 5% solution of purified egg albumin subcutaneously in the right abdominal region (a standardized inflaming procedure, 5). An amount of hormone corresponding to 200,000 c/m (S.A. 1.467 mc/m. mole) was injected through the tail vein. The animals were sacrificed in pairs at intervals of 10, 20, 40, 60 and 100 minutes after the administration of the hormone. Duplicate samples of blood, inflamed tissue, and non-inflamed tissue from the opposite side were plated on aluminum discs and the relative activity (c/m/mg.) determined in a windowless Geiger flow counter. Each sample was corrected for self-absorption and counted for a sufficient length of time to give 5% error or less (6). Immediately after counting, the plated tissues were extracted with a mixture of chloroform: MeOH (1:1), evaporated under nitrogen, and chromatographed on paper by the Zaffaroni method (7), employing the hexane-formamide and chloroform-ethyl acetate-formamide systems. The method used for the identification of the radioactive steroids is described elsewhere (8).

The paper chromatograms were viewed under a Haines-type scanner (9) which defines areas that absorb ultraviolet light at approximately 230–260 mμ. The triphenyltetrazolium chloride (TPTZ) reaction (7) was used to identify the primary alpha-ketol side chain. The Porter-Silber reaction was employed to confirm the presence of a 17,21-dihydroxy, 20-ketone side chain (10). For the determination of the alpha-, beta unsaturated ketone in ring A of steroids the samples were eluted in methanol and read by ultraviolet spectroscopy with a Model DU Beckman using silica cells of 0.5 ml. volume and a 1-cm. light path. When steroids are dissolved in concentrated sulfuric acid, the mixture gives a characteristic chromogen spectrum, which has previously been described in detail (11). An hydrolysis with beta-glucuronidase (Ketodase) was performed to release the glucuronic acid from the steroid molecule using the standard procedures (12).

Two types of derivatives were formed. Acetylation was done by adding to the steroid 3 drops of acetic anhydride and 2 drops of pyridine, leaving the mixture at room temperature over night. Oxidation was performed by adding 0.5 mg. of chromic oxide and 10 drops of glacial acetic acid and leaving the mixture over night at room temperature. The reaction was stopped with cold water and the material was extracted with ethyl acetate.

RESULTS

The amount of radioactivity of the blood dropped rapidly after the first 20 minutes from a maximum of 38.6 c/m/mg. to 3.5 c/m/mg. at 100 minutes (Table 1). The normal connective tissue showed a slight increase from 7.3 c/m/mg. at 10 minutes to 12 c/m/mg. at 40 minutes, then decreased to 3.1 c/m/mg. at 1 hour, and dropped to 1.6 c/m/mg. over the next 40 minutes. The inflamed connective tissue showed an initial level at 10 minutes of 20.1 c/m/mg.; the level increased rapidly to 38.8 c/m/mg. at 40 minutes which was essentially the same count as was present in the blood immediately after injection. This level was 11 c/m/mg. above that found in the blood at the same time; it then decreased to 7.0 c/m/mg. at 100 minutes (Table 1).

The condition of the inflamed connective tissue at 40 minutes was very edematous; however, 100 minutes after hormone injection the tissue showed essentially the same gross appearance as normal tissue.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY AFTER INJECTION OF 4-C^{14} CORTISOL IN ADRENALECTOMIZED MICE

Tissue	Minutes				
	10	20	40	60	100
Blood	*38.6 (35.3–42.0)	38.2 (31.0–45.5)	27.8 (26.6–29.1)	18.3 (11.0–25.6)	3.5 (2.9–4.2)
Normal connective tissue	7.3 (7.3–7.3)	10.4 (8.2–12.7)	12.0 (11.3–12.8)	3.1 (1.1–5.2)	1.6 (1.5–1.8)
Inflamed connective	20.1 (16.2–24.0)	31.7 (22.7–48.5)	38.8 (33.6–44.0)	31.9 (27.2–34.4)	7.0 (3.4–10.5

* These figures represent relative specific activity (c/m/mg.) of dry tissue.

The difference in hormone concentration per milligram of tissue was obtained by calculating the difference in relative specific activity between normal and inflamed connective tissue. The highest difference was found from 40 to 60 minutes after the injection: at this time the difference in concentration of hormone was approximately 0.006 μg./mg. of tissue.

Isolation of Metabolic Products

The blood samples and normal and inflamed connective tissue samples were separately chromatographed on paper. In the blood 3 peaks of radioactivity were found (Fig. 1) and identified as cortisol, tetrahydro cortisol, and tetrahydro cortisol glucuronide. Small amounts of other conjugated compounds were isolated which are not as yet identified.

Identification. The specific activities of the compounds were determined by methods described in detail elsewhere (8) and briefly summarized here.

Cortisol. An aliquot of the radioactive unknown was mixed with 200 μg. of the known non-radioactive cortisol and chromatographed in the chloroform formamide system. Both radioactive peak and known cortisol ran in the same position, with a specific activity of 16.1 c/m/μg. The mixture

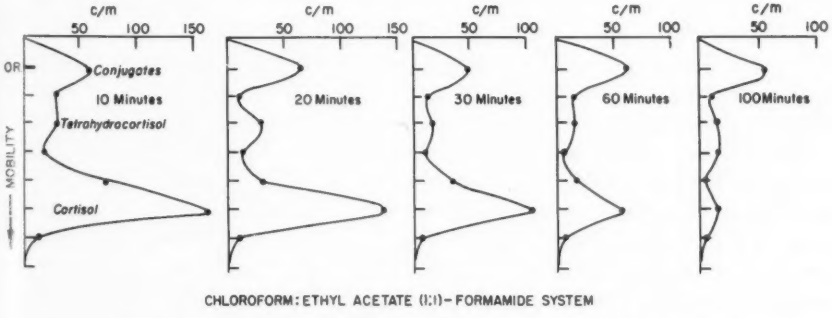


FIG. 1. Chromatographic distribution of steroids in blood after injection of cortisol 4C^{14} in adrenalectomized mice.

was eluted in methanol, acetylated, and run again in the benzene formamide system for 18 hours. Again the radioactivity and known compound were in the same position, having a specific activity of 16.3 c/m/μg. The mixture was further oxidized and chromatographed in the same system for 12 hours; the radioactivity and the carrier had a specific activity of 16.0 c/m/μg. The latter mixture was identified as cortisone acetate. Further proof that the last derivative was cortisone acetate is ascertained from the facts that the mixture was TPTZ positive, gave a maximum at 240 mμ in methanol and sulfuric chromogen gave maxima at 283, 340 and 415 mμ.

Tetrahydro cortisol (*pregnan 3-alpha, 11-beta, 17-alpha, 21-tetrol, 20-one*). Identification of this compound was based on the following data: An aliquot of the radioactive unknown was mixed with 200 μg. of known non-radioactive tetrahydrocortisol and run in the chloroform-ethyl acetate formamide system. Both radioactivity and known compound occupied the same position, having a specific activity of 12.1 c/m/μg. The mixture was then acetylated and rechromatographed in the benzene system for 7 hours; again radioactivity and known steroid had a specific activity of 12.4 c/m/μg. Subsequently, the mixture was oxidized and run in the benzene system for 4 hours, having a specific activity of 12.2 c/m/μg. This derivative was identified as tetrahydro cortisone 3,21 diacetate, which gave a positive reaction with TPTZ, a Porter-Silber positive, and no absorption at 240 mμ in methanol. The sulfuric acid spectrum gave maxima at 335 and 410 mμ, identical with known tetrahydro cortisone diacetate.

Tetrahydro cortisol glucuronide. This compound did not deviate from the origin of the paper chromatogram, even after it was kept for 48 hours in the chloroform-ethyl acetate-formamide system. After elution a beta-glucuronidase hydrolysis was performed and the extract chromatographed in the same system, demonstrating that a compound with the Rf of tetrahydro cortisol had appeared. The procedure for identification of this compound was the same as previously described; the identity of tetrahydro cortisol was thus confirmed. The position of attachment of the glucuronic acid was not determined. Other conjugates were present, since some radioactivity remained near the origin of the chromatogram.

The amounts of the three radioactive steroids in the blood were determined at each of the time intervals given above (Fig. 1). Cortisol decreased progressively with time; tetrahydro cortisol maintained itself at a fairly constant level; and the conjugates including tetrahydro cortisol glucuronide increased with time (Figs. 1 and 2) and came into equilibrium from 40 to 60 minutes. Due to the fact that there were small amounts of connective tissue available, it was necessary to aggregate in separate batches all the samples of inflamed and of non-inflamed tissue for identification. Two radioactive zones were found which correspond to cortisol and the conjugates (Fig. 3). The total amounts of cortisol and conjugates was greater

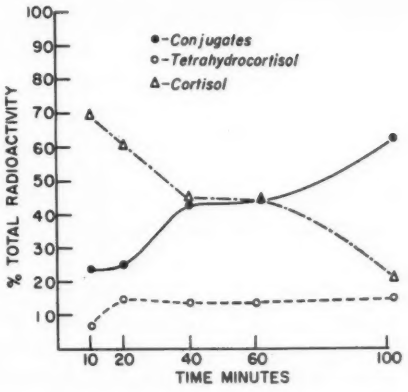


FIG. 2. Percentage of total radioactivity from the different chromatographic peaks. Variations in percentage distribution of cortisol, tetrahydrocortisol and conjugates in the blood of mice at various times following intravenous administration of 4-C^{14} cortisol.

in the inflamed connective tissue, although the percentages found in these two radioactive zones were in the proportion of 1:1 for the inflamed and non-inflamed tissues. The proportions of free cortisol, tetrahydro cortisol and conjugates in the tissues were the same at any given time as those in the blood (Figs. 1 and 3).

DISCUSSION

The experiments demonstrated that loose connective tissue subjected to a topical inflaming stimulus, in adrenalectomized animals which received 4-C^{14} labeled cortisol concentrated the hormone to a greater extent than comparable amounts of non-inflamed connective tissue taken from similar but non-injured areas of the same animal. In view of the fact that cortisol was administered intravenously at the same time that inflammation was induced by a stimulus standardized in this laboratory (5), it appears that the antiphlogistic hormone is attracted in greater quantity to a site of inflammation. However, the data presented here tend to indicate that

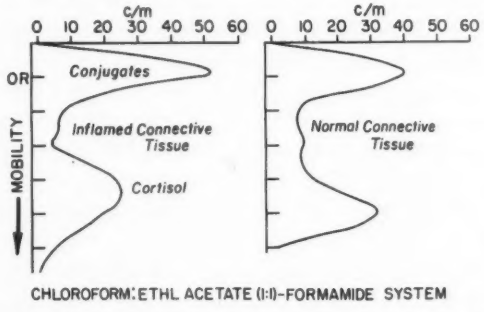


FIG. 3. Chromatographic distribution of steroids in normal and inflamed connective tissue after injection of cortisol 4C^{14} in adrenalectomized mice.

there are no unique mechanisms for attracting the localizing cortisol (trapping effect) from the blood to an inflamed area since the rates of increase and decrease of cortisol in inflamed and non-inflamed tissue were the same. This point is further emphasized by the fact that the ratios of cortisol to the conjugates were the same in inflamed and non-inflamed tissues of the same animals (Fig. 3). Thus, the influx of free and conjugated hormone from blood to tissue was in the same concentration. It appears from previous work that the liver is the major site for conjugation of steroid hormones (13), since there is no conjugation of these substances in hepatectomized animals (14, 15). Therefore, the conjugated steroid present in the tissue must be derived from the blood.

The compounds isolated from blood, inflamed and normal tissues were essentially the same and were found in similar ratios although the total amount of radioactivity was different in each case. The blood level had already started to drop before the inflamed connective tissue had reached its maximum (table 1), and only a small increase was observed in the normal connective tissue.

This indicates that the mechanism of accumulation of cortisol in areas of inflammation is similar to that reported for other substances (16), and increased capillary permeability and edema formation may be important factors. It has been previously shown (1–4) that cortisol decreases markedly the cellular destruction present during inflammation, and in these experiments a decrease in edema was seen to follow closely the administration of cortisol.

The maximum concentration of cortisol in either inflamed or non-inflamed connective tissue occurs at a later time (40 minutes) than the maximum concentration in blood (0–20 minutes). The amount of hormone decreases in normal tissue to a minimal level by the time the half life of the hormone in blood is reached. The amount of hormone, although it is decreasing, is greater per unit amount in the inflamed tissue than in the blood. The concentration of all tissues is minimal at 100 minutes.

The equilibrium in blood between the conjugates and cortisol is maximal between 40 and 60 minutes. The maximum conversion to glucuronides begins at the period corresponding to the greatest concentration of cortisol in the connective tissue. It may thus be hypothesized that the mechanisms by which cortisol exerts anti-inflammatory effects are influenced within the first 40 minutes after it is introduced. Following this period the blood and tissue come into equilibrium for both the conjugated and nonconjugated cortisol (Fig. 2).

Attention has been directed to the fact that inflammation was suppressed by the dose of hormone administered, which is in agreement with our previous investigations (5). The actual amount of hormone required to inhibit inflammation can be deduced from the fact that the differences in amount of radioactive substances between normal and inflamed connective tissue

was 0.006 μg./mg. of tissue. Since half of this amount is cortisol and the conjugates do not have anti-inflammatory potency (5), it is apparent that 0.003 μg./mg. of tissue is the maximum amount which suppressed inflammation in the present experiments. A small portion of the radioactive steroid included some metabolic products not identified here. This will be the subject of a later communication.

The site of hormone deposition in the connective tissue is of some interest. Elsewhere (5, 17) it has been demonstrated that radioactive cortisol tends to localize mainly either within or at the surface of fibroblasts. This cell type comprises 90% of the cells of loose connective tissue of mice (1), and since little hormone was observed in fat or other cells (17), it seems likely that most of the hormone localized here was bound to fibroblasts. The destruction of this particular cell has been thought to be the trigger mechanism of the inflammatory response (2, 18), and other studies (5) have shown that fibroblasts treated with cortisol round up and in this state resist destruction in areas of inflammation. It has been suggested that this increased resistance to cellular damage interrupts a chain reaction of cellular destruction which potentiates inflammation (2, 18).

The evidence presented here indicates that the presence of cortisol for a prolonged period within the area is not necessary to inhibit inflammation since it reaches minimal value at 100 minutes. It has been demonstrated many times (2) that inflammation is suppressed in adrenalectomized animals for prolonged periods (24 hours and more) following a single injection of cortisol. Further, other experiments demonstrate that metabolic products of radioactive cortisol can be recovered quantitatively in excretory products within 24 hours (19). Thus it appears that cortisol tends to enter inflamed tissue in a non-specific manner, produces its anti-inflammatory effects, comes into equilibrium with blood and then is excreted. Since inflammation continues to be suppressed after the hormone concentration of connective tissue decreases, it is probable that cortisol induces alterations in local tissue elements which, in turn, are in such state as to resist the continuation of the inflammatory response. This deduction is supported by histochemical data presented elsewhere (17). It is apparent that there is no degradation of the cyclopentane perhydro phenanthrene nucleus (gonane nucleus) during the period when cortisol exerts its anti-inflammatory effect. In this sense there is no "utilization" of the ring structure in inflamed tissue. Other studies are designed to investigate the relationships between the metabolites of cortisol and the anti-inflammatory effect.

The metabolism of cortisol is very rapid and resembles very much that reported in the human (19). The free steroid had a half-life of 60 minutes in the blood in our experiments with the formation of tetrahydrocortisol and its glucuronide as major derivatives. The half-life in human blood is 70 minutes (20). Another compound obtained after beta-glucuronidase hydrolysis was not fully identified, but resembled beta-cortol (21) in its

chromatographic characteristics. This compound is also a metabolite of cortisol in humans (19).

REFERENCES

1. Dougherty, T. F.: In: Adrenal Cortex. Transactions of the Second Conference. Josiah Macy, Jr. Foundation, New York, 1950, p. 88–114.
2. Dougherty, T. F.: Progress in Allergy, S. Karger, Basel-New York, 1954, Vol. 4, p. 319.
3. Dougherty, T. F.: In: *Recent Progress in Hormone Research* **7:** 307–330. 1952.
4. Hollander, J. L., E. M. Brown, Jr., R. A. Jessar and C. Y. Brown: *J.A.M.A.* **147:** 1629. 1951.
5. Dougherty, T. F. and G. S. Schneebeli: *N. Y. Acad. Sci.* **61:** 328–346. 1955.
6. Berliner, D. L. and W. G. Wiest: *J. Biol. Chem.* **221:** 449. 1956.
7. Zaffaroni, A.: *Recent Progress in Hormone Research* **8:** 51. 1953.
8. Berliner, D. L. and H. A. Salhanick: *Anal. Chem.* **28:** 1608. 1956.
9. Haines, W. U.: *Recent Prog. Hormone Res.* **7:** 255. 1952.
10. Porter, C. C. and R. H. Silver: *J. Biol. Chem.* **185:** 201. 1950.
11. Zaffaroni, A.: *J. Am. Chem. Soc.* **72:** 3828. 1950.
12. Mason, H. L.: *Recent Prog. Hormone Res.* **9:** 301. 1954.
13. Dorfman, R. I.: "The Hormones," V.III, Acad. Press, New York **589:** 664. 1955.
14. Berliner, D. L. and W. G. Wiest: *Fed. Proc.* **14:** 181. 1955.
15. Berliner, D. L., B. I. Grosser, and T. F. Dougherty: *Arch. Biochem. and Biophys.* (in press)
16. Menkin, V.: New Concepts of Inflammation, published by Charles Thomas, 1950.
17. Dougherty, T. F., R. Bigler, G. L. Schneebeli and H. A. Salhanick: *Ann. N. Y. Acad. Sci.* V. **64,** Art. 4, pp. 466. 1956.
18. Eyring, H. and T. F. Dougherty: *Amer. Scientist* **43:** 457–467. Jl 1955.
19. Berliner, D. L. and M. L. Berliner: *Fed. Proc.* **16:** 153. 1957.
20. Migeon, C. J., A. A. Sandberg, H. A. Decker, D. F. Smith, A. C. Paul and L. T. Samuels: *J. Clin. Endocrin. & Metab.* **16:** 1137. 1956.
21. Fukushima, D. K., N. S. Leeds, H. L. Bradlow, T. H. Kritchevsky, M. B. Stoken and T. F. Gallagher: *J. Biol. Chem.* **212:** 449. 1955.

THYROID IODINE METABOLISM FOLLOWING PARTIAL THYROIDECTOMY IN THE RAT[1]

SEYMOUR REICHLIN

Departments of Psychiatry and of Medicine, Washington University, St. Louis, Missouri

ABSTRACT

Compensatory thyroid hypertrophy, and thyroid iodine metabolism have been studied following hemi- and three-quarter thyroidectomy in the rat. Animals have been studied at different time intervals and at two environmental temperatures, 21° and 26° C. The increase in thyroid remnant weight which follows partial thyroidectomy is accompanied by an increase in iodide-trapping ability (thyroid/serum ratio), and a more rapid turnover of iodine in the thyroid gland. These changes appear to be a good measure of activation of the thyroid remnant after partial thyroidectomy.

DURING the course of a study of hypothalamic influences on the pituitary-thyroid axis it became necessary to standardize techniques in the rat for the measurement of the pituitary thyrotrophic hormone (TSH) response to lowered blood thyroid hormone levels. The classical method for studying this response is the demonstration of regeneration of the thyroid gland after partial thyroidectomy. In the following experiments, this method has been further elaborated by measuring certain functional changes in thyroid iodine metabolism in the regenerating thyroid remnant. This paper is concerned with the effect of partial thyroidectomy in the normal rat on the I^{131} uptake, the rate of I^{131} release, and the thyroid/serum iodide ratio. A preliminary report illustrating the use of certain of these methods in the study of rats with hypothalamic lesions has been published (1).

METHODS

Male rats of the Sprague-Dawley strain, weighing between 130 and 480 gm. were used. Purina Laboratory Chow and tap water were given *ad libitum*. The animals were housed in a controlled temperature room which was illuminated by constant periods of light and darkness. Increase in I^{131} release rate following partial thyroidectomy was most readily demonstrable in animals maintained at $26 \pm 1°$ C, and this temperature was used in experiments in which I^{131} release rates were measured. In experiments on changes in thyroid-iodide serum ratio the animals were maintained at $21 \pm 1°$ C before and after operation in order to provide stimulus to regeneration greater than that seen in animals maintained at 26° C.

Received September 12, 1957.

[1] This work was supported in part by U. S. Public Health Service Grant B 834 C and by a grant from the Commonwealth Fund, and was carried out during the tenure of a Lowell M. Palmer Fellowship.

Thyroid I^{131} Release Rate:

Thyroid I^{131} release rates were determined by twice daily counts of neck radioactivity begun 24 to 48 hours after the intraperitoneal injection of 20 or 40 μC of I^{131}. The technique used for neck counting has been described previously (2). Although non-thyroid neck radioactivity makes up less than 5% of total neck radioactivity at 48 hours in normal animals, it was necessary to utilize a correction factor to determine release curves in animals that had been subjected to removal of three-fourths of the thyroid gland because of the low total thyroid iodine content in relation to the non-thyroid neck radioactivity. The correction was also applied to the control and hemithyroidectomized groups in the post-operative curve. The factors were derived from serial observations of the neck counts of a group of seven totally thyroidectomized rats.

I^{131} Uptake:

I^{131} uptake was measured at intervals of 6, 12, 24 and 48 hours after the intraperitoneal injection of 40 μC of carrier free I^{131}, and was expressed as a percentage of the administered dose, an aliquot of which was counted in the same position in relation to the counter as the thyroid gland of the animals studied. The values so obtained measure quantitative differences in uptake, but do not give a measure of the absolute amount of I^{131} in the gland since correction for back-scatter has not been made. Correction for non-thyroid neck radioactivity was required at early intervals after I^{131} administration and this was derived in a preliminary experiment by counting neck radioactivity immediately after thyroidectomy in groups of six rats each, a group being sacrificed at each of the above time intervals after I^{131} administration.

Thyroid/Serum Iodide Ratio:

The thyroid/serum iodide ratio (T/S ratio) was measured utilizing the method of VanderLaan and Greer (3). Two hours before death the animals were injected intraperitoneally with 6 mg. of n-propylthiouracil[2] in 1.0 ml. of 0.01 N NaOH in physiological saline solution. One hour later 5 μC of carrier free I^{131} was injected subcutaneously. At the time of sacrifice neck blood was obtained by decapitation and the thyroid gland was cleaned and weighed to 0.1 mg. on a torsion balance. The radioactivity of the thyroid gland and of 0.1 ml. of serum were determined in a well type scintillation counter and the T/S ratio calculated from these data.

Partial Thyroidectomy:

This operation was carried out under ether anesthesia, with atropine premedication (0.4 mg.) in both thyroidectomized and sham operated controls. In the hemi-thyroidectomy procedure the left lobe of the thyroid gland was dissected free from the trachea, trimmed and weighed to 0.1 mg. on a torsion balance. The parathyroid gland was left attached to the trachea. In the operation of three-quarter thyroidectomy the left lobe was removed and weighed, and the lower half of the right lobe removed and weighed. An estimate of the weight of the residual tissue could be made by subtracting the weight of the removed portion of the right lobe from the weight of the left lobe. In sham operated controls, the thyroid gland was mobilized and the isthmus severed.

RESULTS

I. The effect of partial thyroidectomy on I^{131} release rate

a. The immediate effect of hemi-thyroidectomy on I^{131} release. Following an initial control period of observation of the I^{131} release rate in 17 rats the left

[2] Obtained through the kindness of Dr. Stanton M. Hardy, Lederle Laboratories

lobe of the thyroid gland was removed. The "release curve" was subsequently followed until counting rates fell to below twice background levels. Two of the 17 hemi-thyroidectomized rats developed local hematomata and one a local abscess, and these were discarded from the series. Four additional rats were subjected to sham thyroidectomy.

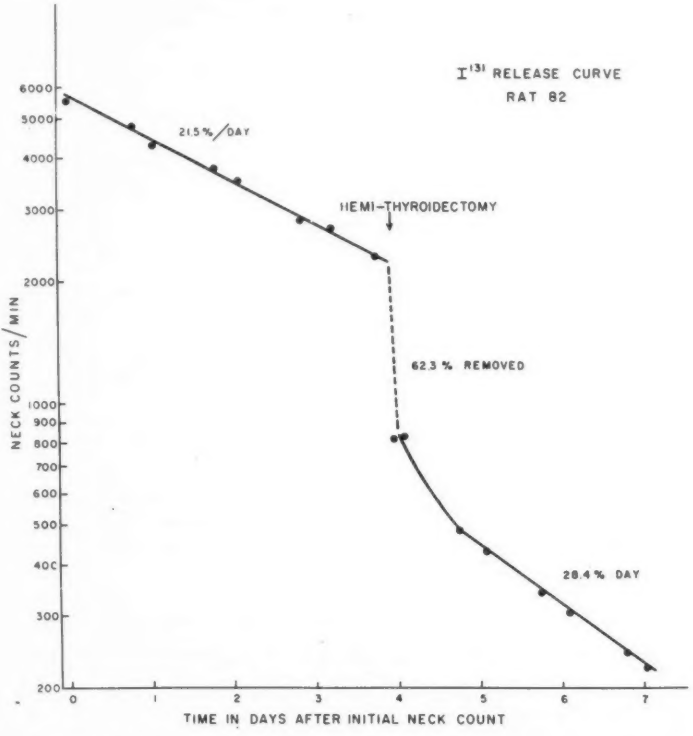


FIG. 1. Removal of the left lobe of the thyroid gland during the course of a release curve. By measurement of neck radioactivity before and after the thyroidectomy the removed portion was found to be 62.3% of the total gland content. An initial acceleration of release within the first post-operative day was observed.

The immediate effects of hemi-thyroidectomy on I^{131} release rates were variable. In each of three animals, a marked increase in the rate of release occurred during the first post-operative day (Fig. 1). Thereafter, continued more rapid release rate was noted in one of the three, the other animals showing either no change or a slowing of rate, as compared with pre-operative levels.

In six animals, sustained increase in I^{131} release rate apparently began within the first post-operative day (Fig. 2). The average ratio of post-operative to pre-operative slopes in the group of nine animals showing

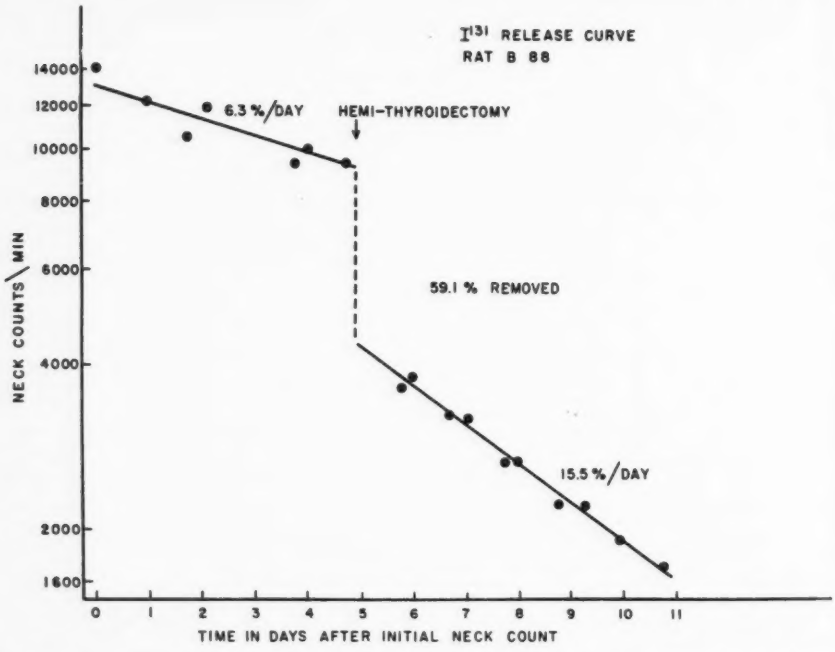


FIG. 2. Removal of the left lobe of the thyroid gland during the course of a release curve. By measurement of neck radioactivity before and after thyroidectomy the removed portion was found to 59.1% of the total gland. A sustained increase in release rate was noted beginning within the first postoperative day.

acceleration was 1.32. There was no change in slope following hemi-thyroidectomy in three rats (Fig. 3), and in two others, the slope of the post-operative release curve was slower than the pre-operative curve (post-operative to pre-operative slope ratio 0.87). Nine of the fourteen rats therefore gave evidence of increased rate of I^{131} release beginning within 24 hours of hemi-thyroidectomy.

Sham thyroidectomy exerted no effect on release rate in three of the four rats, and in the fourth animal a two-day period of thyroid inhibition occurred, followed by a return to a release rate that was slightly faster than that of the control period.

b. Late changes in I^{131} release rates following partial thyroidectomy. Radioiodine release curves were studied in three groups comprising seven rats each before and eight weeks after sham thyroidectomy, hemi-thyroidectomy or three-quarter thyroidectomy (Fig. 4). Curves plotted by the method of least squares from the average of neck counts indicated an increase of 9.2% (18.4 to 20.1% per day) in post-operative slope in the sham thyroidectomized animals, an increase of 20.6% (from 21.9 to 26.4% per day) in the hemi-thyroidectomized animals, and in the three-quarter thy-

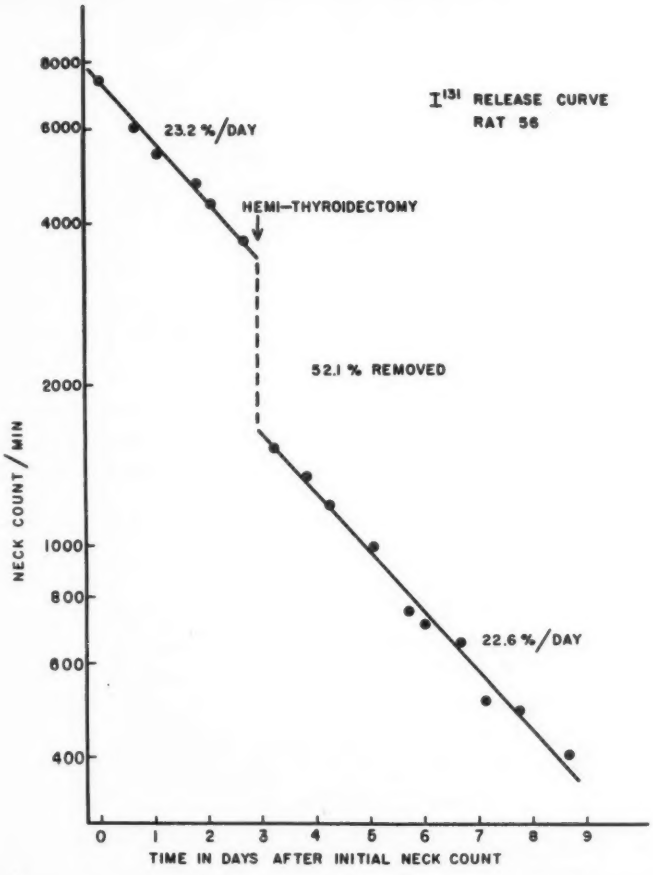


FIG. 3. Removal of the left lobe of the thyroid gland during the course of an I^{131} release curve. No difference between pre- and post-operative slopes was observed in this animal.

roidectomized animals an increase of 35.5% (from 21.1 to 28.6% per day). In considering changes in the slopes of individual animals it should be pointed out that increase in release rate occurred in every individual subjected to either hemi- or three-quarter thyroidectomy and this increase varied between 15% and 154%. On the other hand, only one of the control animals showed an increase in slope that was greater than 15% (42%), the rest not exceeding an 8% increase over baseline levels.

II. The effect of partial thyroidectomy on thyroid I^{131} uptake

Thyroid radioiodine content at intervals of 6, 12, 24 and 48 hours after intraperitoneal injection of 40 μC of carrier-free I^{131} was determined in the three groups of animals described in the preceding paragraph

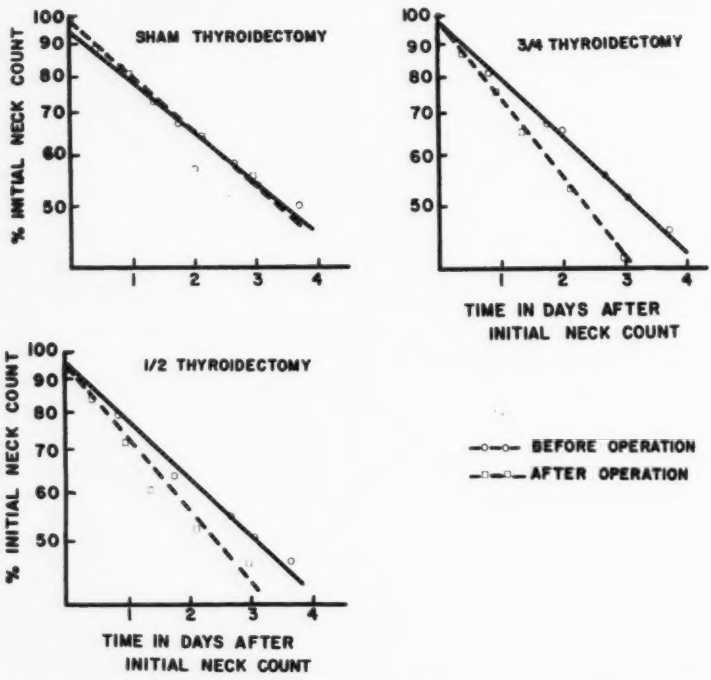


FIG. 4. The effect on I^{131} release rates of removal of one-half or three-quarters of the thyroid gland of the rat. Each group consisted of 7 animals, and release rates were determined immediately before and 8 weeks after operation. Lines fitted by method of least squares.

(Table 1). These animals were studied 8 weeks after operation. It was noted that the highest thyroid radioactivity was found at 24 hours in the control animals (differences between observations at 12, 24, and 48 hours significant at 99% level of probability). Peak levels occurred at 12 hours in both the hemi-thyroidectomized and three-quarter thyroidecto-

TABLE 1. THYROID I^{131} UPTAKE IN PARTIALLY THYROIDECTOMIZED RATS

Group	Number of animals	Thyroid I^{131} content (% administered dose)			
		6 hr.	12 hr.	24 hr.	48 hr.
Control	7	9.4	13.5	*14.8*	12.3
½-Thyroidectomy	7	7.6	*11.4 ± 0.9**	10.8	8.5
¾-Thyroidectomy	7	3.0	*4.4 ± 0.9***	4.1	3.0

Italicized values are the highest radioiodine contents noted.

* Differences between thyroid I^{131} content in control group at all time intervals significant at $P < 0.05$.

** Differences between thyroid I^{131} content in both hemi-thyroidectomized and ¾-thyroidectomized at 12 and 24 hours not statistically significant.

TABLE 2. COMPENSATORY HYPERTROPHY AND THYROID/SERUM IODIDE RATIO CHANGES FOLLOWING ¾-THYROIDECTOMY

Time after partial thyroidectomy (days)		Number of animals	Body weight, gm.		Weight of thyroid remnant, mg. ± S.E.		Thyroid remnant weight/body weight ratio, mg./kg. ± S.E.		Thyroid: serum I^{131} ratio ± S.E.
			Initial	Final	Initial	Final	Initial	Final	
15	Control	3	138	218	—	18.0 ± 0.1	—	0.83 ± 0.04	18.4 ± 3.7
	Thyroidectomized	4	134	193	3.3 ± 0.5	8.1 ± 2.1	0.24 ± 0.03	0.40 ± 0.07	56.4 ± 11.2
30	Control	5	143	295	—	19.3 ± 1.3	—	0.66 ± 0.05	29.0 ± 2.1
	Thyroidectomized	5	140	196	1.9 ± 0.4	10.2 ± 0.8	0.14 ± 0.03	0.43 ± 0.05	38.7 ± 9.6
43	Control	5	142	328	—	22.4 ± 0.7	—	0.68 ± 0.03	33.7 ± 4.5
	Thyroidectomized	6	135	302	2.6 ± 0.4	8.6 ± 1.2	0.20 ± 0.03	0.29 ± 0.05	50.3 ± 8.8

mized groups (differences between observations at 12 and 24 hours not statistically significant; between 24 and 48 hours significant at 99% level of probability). At eight weeks, although there had been relative increases in radioiodine uptake in the hemi-thyroidectomized animals this had not reached control levels. The radioiodine uptake of the three-quarter thyroidectomized animals was only 30% of that of the control group.

III. The effect of partial thyroidectomy on thyroid/serum iodide ratio

In this experiment groups of rats were subjected to three-quarter thyroidectomy or to sham thyroidectomy and kept at a temperature of 21 ± 1° C. At intervals of 15, 30 and 43 days the animals were sacrificed and the T/S ratio determined (Table 2). It is apparent that significant increase in T/S ratio occurred in groups studied at all three intervals. There did not appear to be a tendency for progressive increase in iodide-trapping ability with time between two and six weeks. The average T/S ratio of the partially thyroidectomized animals was 48.0 ± 5.5 S.E. in contrast to a T/S ratio of 28.4 ± 2.5 S.E. in the controls, representing a highly significant increase of 69% over control levels.

IV. Compensatory hypertrophy following partial thyroidectomy

Data are available from two experiments. These are in animals subjected to hemi- and three-quarter thyroidectomy, maintained at 26° C, and sacrificed at ten weeks (Table 3) and in rats subjected to three-quarter

TABLE 3. COMPENSATORY HYPERTROPHY TEN WEEKS FOLLOWING PARTIAL THYROIDECTOMY IN THE RAT

Group	Number of animals	Body weight (gm.)		Weight thyroid remnant (mg. ± S.E.)		Thyroid remnant weight/body weight (mg./kg.)	
		Initial	Final	Initial	Final	Initial	Final
Sham Thyroidectomy	7	302	442	—	25.7 ± 1.8	—	0.58
½ Thyroidectomy	7	284	430	9.1 ± 0.8	14.7 ± 0.6	0.32	0.34
¾ Thyroidectomy	7	286	392	3.7 ± 2.2	7.4 ± 1.8	0.13	0.19

thyroidectomy, maintained at 21° C, and sacrificed at approximately two-week intervals up to six weeks following the operation (Table 2). In the first experiment (Table 3) there was a 61% increase in thyroid remnant weight in the hemi-thyroidectomized animals, and a 100% increase in remnant weight in the three-quarter thyroidectomized animals. If one estimates the initial total thyroid weight in the control animals from observations of initial hemi-thyroid weight in the hemi-thyroidectomized animals (18.2 mg.) there appears to have been a 41% increase in thyroid size in the control group. In neither group of partially thyroidectomized animals did the final weight of the remnant approach the total weight of the thyroid glands of control animals. Estimations of compensatory hypertrophy based on thyroid weight/body weight ratios before and after operation gave less clear evidence of relative hypertrophy in the hemi-thyroidectomized animals (0.32 mg/Kg. vs. 0.34 mg./Kg) than they did in the three-quarter thyroidectomized animals in which this ratio rose from 0.13 to 0.19 mg./Kg., a change of 46%.

In animals maintained at 21° C there is clear evidence of increased thyroid size both absolute and relative at all three intervals following partial thyroidectomy. The maximum relative increase in remnant size was noted in animals sacrificed at 30 days (207%), but there are too few animals in each group to be certain that this time interval is associated with the maximum change in relative thyroid size.

DISCUSSION

These experiments have outlined certain changes in thyroid iodine metabolism which occur in the thyroid remnant following partial thyroidectomy.

Within the first day of hemi-thyroidectomy an increase in rate of radioiodine release from the thyroid remnant was observed. Non-specific effects of operative stress can be excluded as a cause of this response since it did not occur in sham operated animals, does not occur following laparotomy in the rat (unpublished observations), and does not occur in rabbits subjected to surgical trauma (4). It is probable that this phenomenon is a reflection of increased TSH release in response to the direct or indirect effects of lowered blood thyroid hormone levels which are in turn consequent upon the removal of half of the functioning thyroid tissue.

Considerable interest is attached to the observation that apparent increase in TSH release was noted within 24 hours of hemi-thyroidectomy. Logothetopoulos and Doniach (5) employing histological criteria, have also found evidence of increase in TSH release within 3 days of a similar procedure.

Although early acceleration of I^{131} release rate occurred in the majority of normal animals studied, the fact that a third failed to show this response makes it a relatively unreliable test of the pituitary response to lowered

blood thyroid hormone levels. Some of the factors interfering with the effect may have been the associated trauma with its non-specific depressing effect on thyroid function, differences in peripheral turnover time of thyroxine, or differences in the sensitivity of the mechanisms concerned with the release of TSH.

On the other hand, increase of I^{131} release rate of 15% or more was apparent in all animals subjected either to hemi- or three-quarter thyroidectomy when tested at eight weeks after operation. Only one of the sham operated control animals showed a comparable degree of acceleration, which suggests that the release curve obtained after several weeks is a relatively reliable index of increased thyroid activity and presumably therefore of increased TSH release. A similar acceleration of iodine turnover following partial thyroidectomy has been described previously to occur in the rat (5) and rabbit (6).

In the evaluation of quantitative changes in I^{131} release rates after partial thyroidectomy the influence of partial thyroidectomy upon recirculation of I^{131} from peripherally degraded thyroid hormone must be considered. Decrease in the uptake of iodine through loss of thyroid mass will lead to an observed increase in I^{131} release rate since the observed release rate is related to the actual release rate by the formula

$$R_0 = R_a - (C \cdot R_a)$$

where

R_0 = observed release rate in % per day

R_a = actual release rate in % per day

C = fraction of thyroxine iodine which is taken up by the thyroid gland.

From the data given in this paper it is not possible to calculate precisely the magnitude of the change in observed release rate which is attributable to the change in thyroid iodine uptake. The fraction of I^{131} which is recirculated is not known accurately, and there is a variable degree of increase in iodine uptake with time after partial thyroidectomy. A rough estimate of the magnitude of the change in observed release rate can be derived from data in section Ib and Table 1. In normal animals an observed release rate of 18.4% per day would reflect an actual release rate of 21.6% per day if 0.148 is the fractional recirculation of degraded thyroidal iodine. Following the removal of three-quarters of the thyroid gland (and provided there there was no increase in I^{131} uptake relative to the size of the remnant) an actual release rate of 21.6% per day would give an observed release rate of 20.8% per day instead of the rate of 18.4% which was observed in intact animals. This is a difference of 13% which is considerably less than the 35.5% difference observed in the present studies. Similar calculations, based on expected changes after hemi-thyroidectomy indicate that an observed release rate of 18.4% per day would be expected to increase to 20.0% per day. This difference (8.4%) may be contrasted with the observed ac-

celeration in the hemi-thyroidectomized animals of 30.6%. It is noteworthy that release rates did not increase in proportion to thyroid tissue loss, suggesting inadequate compensatory function.

From the work of Rawson, Sterne and Aub (7) it is at least theoretically possible to attribute the increased I^{131} release rate following partial thyroidectomy to decreased destruction of a constant amount of TSH by a smaller mass of thyroid tissue rather than to increased TSH output. This possibility is not likely for several reasons. It has been shown that the rate of TSH destruction in the whole animal is a function of general body metabolic rate and not of local destruction by the thyroid gland (8, 9). Moreover, in a specific investigation of this point it was demonstrated by Logothetopoulos and Doniach (5) that TSH, when injected into hypophysectomized, hemi-thyroidectomized animals exerted no greater effect on the thyroid remnant than do similar injections into hypophysectomized animals with intact thyroid glands.

In the present studies I^{131} uptake was increased after partial thyroidectomy relative to the amount of tissue left at the time of operation. This was shown in the hemi-thyroidectomized animals (Table 1) in which I^{131} uptake was 75% of the normal amount. This finding is confirmatory of those in the rat (5), rabbit (6) and man (10). The three-quarter thyroidectomized animals did not show this effect to a significant degree, their highest uptake value being only 30% of the normal. This is a discrepant finding in view of the results with hemi-thyroidectomized animals, and those in Part III (Table 2) in which three-quarter thyroidectomy was followed by a striking increase in thyroid size and avidity for iodide. The apparent low uptake is not entirely due to the more rapid turnover of iodine in the gland since the release rates in this group were only moderately increased over the normal. Using a 3-hour determination of thyroid radioactivity, Logothetopoulos and Doniach (5) have found progressive increase in uptake up to 120 days after three-quarter thyroidectomy.

Partial thyroidectomy led to an increase in the avidity of the gland for iodide, typified by an increase in the thyroid/serum iodide ratio. The T/S ratio is clearly influenced by the degree of TSH stimulation (3) and increased TSH stimulation is probably responsible for the changes observed in these experiments. There are other factors, however, independent of TSH stimulation which influence iodide trapping. The existence of these modifying influences has led to criticism of Greer's contention (11) that there are two kinds of thyroid-stimulating substances of pituitary origin, one which influences growth, and the other which influences thyroid iodide trapping (12, 13, 14, 15). In view of the fact that the present experiments were carried out preliminary to experiments on the hypothalamic regulation of the pituitary thyroid axis, it is important to consider whether the increase in T/S ratio which follows partial thyroidectomy can be due to anything but increased stimulation of the thyroid gland.

The critical observation made by VanderLaan and Caplan (13), Halmi and Spirtos (14) and Halmi (12, 15) is that the T/S ratio is also dependent in some way upon the iodine concentration of the thyroid gland, which falls in the presence of iodine deficiency, or after prophylthiouracil administration. Despite the fact that partial thyroidectomy reduces the size of the thyroid iodine pool, the concentration of iodine containing compounds within the remnant is identical with that of the whole gland, and would be expected to have the same avidity for iodine. If the concentration of iodine containing compounds within the regenerating remnant changed, it could be due only to alteration in the steady state of the thyroid gland by an increase of iodine loss over iodine organification, or by an increase in gland size relative to iodine content. In the absence of anti-thyroid drugs, and in the presence of constant high dietary iodine, this alteration in steady state could only arise as a consequence of increased thyroid metabolic activity or growth. Therefore, even if the rise in T/S ratio were merely a consequence of change in some iodine containing compound within the gland, it would still appear to be an index of thyroid activation.

In all experiments, regeneration of the thyroid remnant after partial thyroidectomy was noted. This was apparent even at two weeks in animals maintained at 21° C. This change was to be anticipated from the large number of similar observations in the literature (5, 6, 16, 17).

Acknowledgments

The technical assistance of Mrs. Christa Kindermann is gratefully acknowledged.

REFERENCES

1. Reichlin, S.: *Endocrinology* **60:** 567. 1957.
2. Reichlin, S.: *Endocrinology* **60:** 470. 1957.
3. VanderLaan, W. P. and M. A. Greer: *Endocrinology* **47:** 36. 1950.
4. Brown-Grant, K., G. W. Harris and S. Reichlin: *J. Physiol.* **126:** 29. 1954.
5. Logothetopoulos, J. H. and I. Doniach: *Brit. J. Exp. Path.* **36:** 617. 1955.
6. Johansen, R., R. E. Gardner, M. Galante, F. F. Marchi, T. W. Ledwich, M. H. Soley, K. G. Scott, E. F. Miller and H. J. McCorkle: *Surg. Gynec. and Obst.* **93:** 303. 1951.
7. Rawson, R. W., Grace D. Sterne and J. C. Aub: *Endocrinology* **30:** 240. 1942.
8. D'Angelo, S. A.: *Endocrinology* **56:** 37. 1955.
9. Querido, A., A. A. H. Kassenaar and L. D. F. Lameyer: *Acta Endocrinol.* **19:** 152. 1955.
10. Szilagi, D., J. L. Barrett and L. E. Preuss: *J. Clin. Endocrinol and Metab.* **15:** 1409. 1955.
11. Greer, M. A.: *J. Clin. Endocrinol. and Metab.* **12:** 1259. 1952.
12. Halmi, N. S.: *Endocrinology* **54:** 97. 1954.
13. VanderLaan, W. P. and R. Caplan: *Endocrinology* **54:** 437. 1954.
14. Halmi, N. S. and B. N. Spirtos: *Endocrinology* **55:** 613. 1954.
15. Halmi, N. S.: Ciba Foundation Colloquia on Endocrinology, Little, Brown and Company, Boston, **10:** 79. 1957.
16. Loeb, L.: *J. Med. Res.* **40:** 199. 1919.
17. Uotila, U. U.: *Endocrinology* **26:** 129. 1940.

PATHOLOGIC CHANGES IN SENSITIZED RATS EXPOSED TO STRESS[1]

W. A. J. CRANE AND D. J. INGLE

The Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Illinois

ABSTRACT

Male rats of the Sprague-Dawley strain were subjected to unilateral nephrectomy and were given a high sodium chloride diet to eat *ad libitum*. These sensitized rats were exposed to various stressors (limb ligation once each week, surgery once each week, hemorrhage twice each week, a burn once each week, a fracture once each week, or an injection of 1 per cent formaldehyde twice each day) for 8 weeks. It should be emphasized that each form of stress was near the maximum severity which could be tolerated by the rats. Some pathology occurred in the kidneys of non-stressed sensitized rats and was aggravated by exposure to some of the stressors. Three of the stressed rats had elevated blood pressures. Myocardial changes were seen in only a few of the stressed animals. The pathology caused by these stressors was less severe than that seen in sensitized rats overdosed with hormones and in sensitized rats exposed to cold in other experiments. The hearts and kidneys did not increase in size when the animals were exposed to various stressors, the weights of thymus were moderately suppressed and the weights of adrenal glands were moderately increased.

SELYE'S concept (1) of the adaptation diseases holds that derailment of adaptative mechanisms during exposure to non-specific forms of stress is the principle cause of many of the diseases of man. Emphasis is placed upon the importance of changes in the secretory activity of the hypophysis and of the adrenal cortex in causing disease. Most of the evidence supporting the hypothesis of Selye is based upon the use of experimental animals which have been sensitized by unilateral nephrectomy and by a high dietary load of sodium chloride. There are many studies showing that the overdosage of sensitized and even normal animals with certain steroids or with certain hypophyseal hormones will cause metabolic upsets and organic changes which simulate human diseases (1).

Although the literature on stress has become enormous, there is a paucity of evidence that exposure of normal animals to naturally occurring forms of stress causes disease. Indeed, there are only a few studies showing that exposure of sensitized animals to stress under highly artificial conditions causes pathologic changes. When the sensitized rat is exposed continuously to cold over a period of several weeks, severe renal and some myocardial

Received September 27, 1957.

[1] This work was supported by grants from the American Cancer Society, the United States Public Health Service C-2213(C3), and a grant from The Upjohn Company.

damage results (2, 3, 4). During exposure to cold the animal approximately doubles its caloric intake to meet increased neeeds for heat energy. It is probable that the pathologic changes which appear in the sensitized rat exposed to cold are dependent in part upon the increase in dietary load, especially of sodium chloride. There is one report (5) that exposure of normal rats to cold for 10 to 18 months caused some changes from normal in the hearts and kidneys and a small average rise in blood pressure. Selye (2) has noted some renal pathology in sensitized rats given injections of dilute

TABLE 1. HIGH SALT, MEDIUM CARBOHYDRATE DIET

Constituent	Grams	Constituent	Grams
Cellu flour	60	Mazola oil	190
Salt mixture U.S.P. XIV	40	Casein	160
Dried yeast (Pabst)	100	Starch	300
Wheat germ oil	10	Dextrin	150
Cod liver oil	10	Sucrose	140
Mazola oil plus 100 mg. K	10	Sodium chloride	50

formalin and in similar rats forced to exercise, but a detailed report of results was not published.

In the present study, sensitized rats were exposed to various stressors (limb ligation, shock, surgery, hemorrhage, burns, fractures, and injections of formalin) for a period of 8 weeks. Pathologic changes in the heart and kidney were noted in some animals and in a few the blood pressure was elevated.

METHODS

Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets prior to unilateral nephrectomy. Thereafter, the animals were given a high sodium chloride diet (Table 1). The dry diet was tightly packed into individual Franke animal feeding jars which were weighed every 24 hours.

Six normal rats were maintained on the stock diet. Eight groups of sensitized (unilateral nephrectomy, high salt diet) rats containing six rats per group, were each exposed to one of the following conditions for 8 weeks:

1. Laparotomy was performed under ether anesthesia once each week by opening and closing the abdominal wall from the diaphragm to the level of the bladder. The incision was made in a different site on each occasion.

2. Once each week, a strip of skin approximately 15 mm. wide extending from the neck to the base of the tail was excised from the back under ether anesthesia. After 4 operations, the closure of the wound at each subsequent operation left the remaining skin very tight on the body of the rat, but after a few days of stretching and growth, the skin became loose again.

3. Hemorrhage was done twice weekly by placing each rat in an activity-restriction cage, amputating the tip of the tail and immersing it in a heparin solution until a total of 8 cc. of blood was removed. The loss of this amount of blood always left the rat pale and weak, but no deaths occurred.

4. A burn was caused once each week by immersing the body of the anesthetized rat in water at 70° C for 4–6 seconds. The head, feet and tail were not burned. The time of

exposure for each rat was determined by the severity of the burn the preceding week. The burns were not deep but caused marked hyperemia of the skin and some surface scaling after a few days. Following a burn, each rat became hyper-irritable and fought with its cage mates, although it still permitted handling by the experimenter without forcible restraint.

5. A bone (tibia or femur or humerus or the radius and ulna together) was fractured under ether anesthesia once each week.

6. Ligation of one hind limb was done under ether anesthesia the first week, the opposite limb the second week, and alternately thereafter for 8 weeks. The procedure has been described (6). The ligatures remained in place for 2 hours the first and second week, 2 hours on each of the third and fourth ligations and for 3 hours during each of the last 4 times.

7. A 1% solution of formaldehyde was injected subcutaneously twice each day, 0.5 cc. per injection.

8. Six sensitized rats were maintained under normal conditions for 8 weeks. The blood pressure was recorded in groups 3, 4, 6, and 7 and in the 6 normal rats maintained on the stock diet. The rats were anesthetized with ether and the abdominal aorta was cannulated below the level of the renal arteries. The cannula was attached by a short length of fine-bore rubber tube filled with heparinized saline to a mercury manometer. The record was taken when anesthesia was light as judged by retraction of the rat's hind foot on pinching.

All rats were killed by exsanguination under ether anesthesia. Autopsy was performed and gross pathologic changes noted. The heart, kidney, adrenal glands, and thymus were weighed. The heart, kidney and portions of liver, spleen, pancreas, lung, stomach and bowel were fixed in 10% neutral formalin. Hematoxylin and eosin was the routine stain and selected examples of heart and kidney were examined by the periodic acid-Schiff routine, Mallory's method, and Weigert's resorcin-fuchsin stain for elastic tissue. The adrenal glands were fixed in 1% formol-calcium, cold 10% neutral formalin and cold acetone. Frozen sections of gelatin embedded material were examined with oil red 0 for lipids (7), the Schultz method for cholesterol (8), and with polarized light for birefringent crystals. Acid phosphatase was demonstrated in frozen sections by the method of Gomori (7) and acetone processed material was used for the Gomori alkaline phosphatase procedure (7).

RESULTS

The data are summarized in Table 2. The stressed animals showed a reduction in weight gain over the experimental period, but this varied considerably from one group to another. Their average daily food consumption was slightly less than that of the control rats.

Heart: The average weight of the heart of rats exposed to stress ranged both above and below the mean for the control animals (Table 2). No gross abnormality was seen in any heart. Microscopically the hearts from the control rats and from the majority of the stressed groups were normal (Table 2). Only in some animals stressed by repeated burns or laparotomy was there any cardiac abnormality. This consisted of slight hypertrophy of the cardiac muscle cells with an increase in Anitschkow myocytes situated mainly around blood vessels. In a few instances focal aggregates of cells, predominantly myocytes, were present in the myocardium (Fig. 1) or sub-endocardium. These nodules did not show the classical features of the Aschoff node. No abnormality was detected in the coronary arteries or their

TABLE 2. EFFECTS OF STRESSORS ON UNILATERALLY NEPHRECTOMIZED ADULT MALE RATS EATING 4% NaCl MEDIUM CARBOHYDRATE DIET. AVERAGES AND STANDARD ERRORS FOR 6 RATS PER GROUP

Experiment	Body weight, gm.		Organ weights, mg.				Mm. Hg. blood pres.	Gm. food daily	Ratings* of pathology, average and range		
									Kidney		Heart
	Initial	Final	Heart	Kidney	Thymus	1 adrenal			Gross	Micro	Micro
Control	302	446 ±14.7	1300 ±63	2113 ±94	561 ±36	26.0 ±1.3	124 ±2.7	19.2	0.5 ±0.13 (0–1.0)	0.75±0.35 (0.5–1.0)	0
Laparotomy	302	329 ± 9.0	1230 ±23	1853 ±86	378 ±46	31.0 ±1.3		17.6	0.6 ±0.09 (0–1.5)	2.2 ±0.58 (0.5–3.0)	1.2±0.26 (0–2.0)
Skin Excision	302	357 ± 9.0	1360 ±47	1981 ±72	394 ±42	29.5 ±1.9		16.0	0.58±0.07 (0.5–1.0)	0.9 ±0.24 (0.5–2.0)	0
Hemorrhage	303	400 ± 9.0	1280 ±39	1715 ±57	384 ±23	29.0 ±1.6	125 ±1.9	18.3	0.8 ±0.25 (0.5–1.0)	1.5 ±0.34 (1.0–3.0)	0
Burn	306	374 ±18.4	1425 ±59	2020 ±104	333 ±40	32.7 ±1.7	132 ±6.1	19.0	1.42±0.30 (0.5–2.5)	2.0 ±0.62 (0.5–3.0)	0.7±0.34 (0–2.0)
Fracture	302	372 ± 3.7	1180 ±18	1898 ±94	440 ±42	32.6 ±6.3		15.6	0.5 ±0.13 (0–1.0)	1.0 ±0.33 (0.5–3.0)	0
Limb Ligation	303	347 ±10	1308 ±57	1983 ±64	360 ±32	35.3 ±2.1	136 ±4.6	17.6	1.92±0.15 (1.5–2.5)	1.25±0.25 (0.5–2.0)	0
Formaldehyde	305	433 ±11.1	1233 ±31	1995 ±33	368 ±28	30.3 ±1.9	118 ±5.5	17.9	1.0 ±0.26 (0.5–2.0)	1.1 ±0.20 (0.5–2.0)	0

* The ratings of gross renal pathology are based on the following scale: 0 = no abnormality; 1 = few tiny nodules; 2 = moderately nodular with some mottling; 3 = more nodules, mottling with some gray spots; 4 = rough, mottled, with gray and red spots; and 5 = changes listed previously more severe, giving "flea-bitten" grayish-yellow appearance.
The ratings of the microscopic changes are based upon the following features. KIDNEY: 0 = no abnormality; 1 = hypertrophy of glomeruli and tubules, a few hyaline casts, and slight focal interstitial inflammation; 2 = active pelvic inflammation; 3 = glomerular lesions, no apparent vascular damage; 4 = hyalinization of glomeruli, sclerosis of arterioles; and 5 = necrosis of glomeruli and arterioles. HEART: 0 = no abnormality; 1 = hypertrophy of muscle; 2 = focal inflammation or fibrosis of myocardium; 3 = changes listed previously, sclerosis of vessel walls; and 4 = necrosis of myocardium and/or vessel walls.

branches. Valvulitis was not seen. In 8 rats foci of cartilagenous metaplasia were present in the aorta at the origin of the aortic valve (Fig. 2). This change was not confined to rats of any one group and has been seen in non-stressed rats.

Kidney: The average weight of the kidney from the stressed groups was reduced to a variable degree as compared with the control rats (Table 2). Grossly they showed slight nodularity and mottling with some gray and an occasional red spot on the surface. Microscopically the most severe damage was seen in rats submitted to repeated laparotomy, or burning, and to a less extent hemorrhage (Table 2). Slight

FIG. 1. Heart. Limb ligation. A small fibrous nodule containing Anitschkow myocytes is seen in the interstitial tissue of the myocardium. Hematoxylin and eosin. ×420.

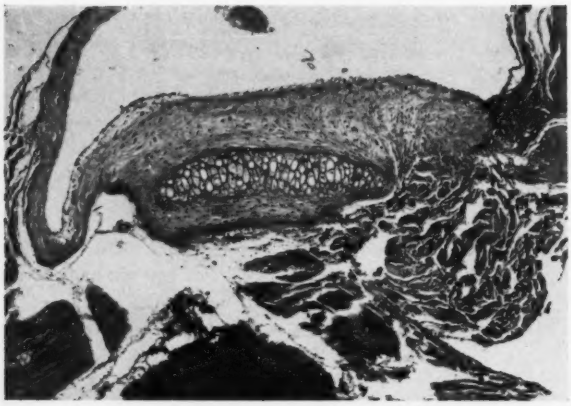


FIG. 2. Aortic valve. Limb ligation. An islet of cartilage encapsuled by fibrous tissue is present at the base of the valve. H and E. ×50.

hypertrophy of glomeruli and tubules was present in some animals of all groups. Commonly this was associated with some focal dilatation of tubules, hyaline cast formation, and with infiltration of a few lymphocytes

FIG. 3. Kidney. Burn. The tubular epithelium shows vacuolation and hyaline droplet change. Piero-Mallory. ×192.

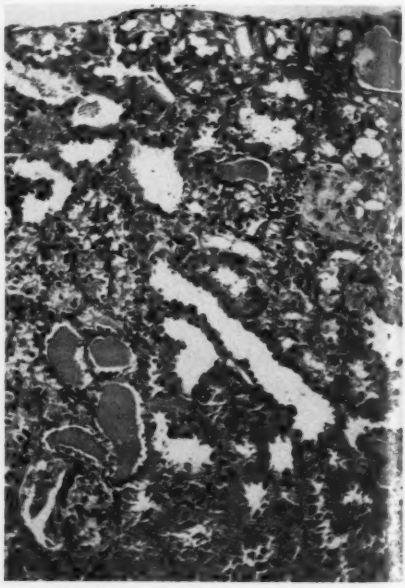


FIG. 4. Kidney. Burn. There is glomerular hyalinization, irregular tubular dilatation, and hyaline cast formation. H and E. ×125.

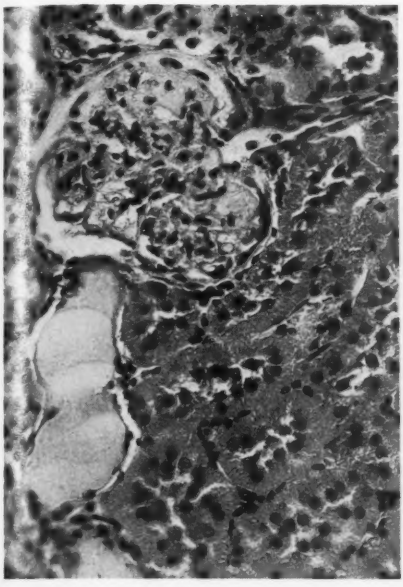


FIG. 5. Kidney. Laparotomy. Early hyalinization is seen at the vascular pole of the glomerulus and the proximal tubule is filled with a hyaline cast. The afferent arteriole shows no arteriolosclerosis. H and E. ×255.

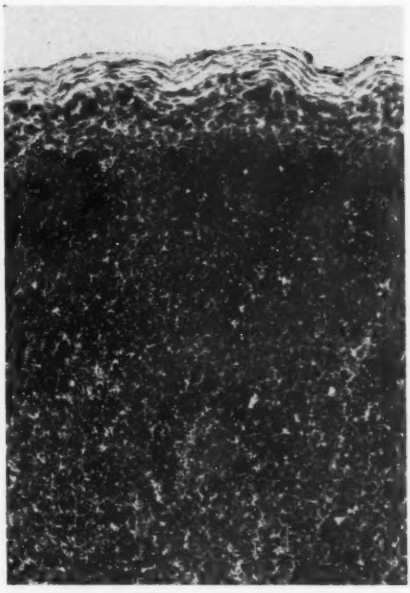


FIG. 6. Adrenal. Control rat. Zona glomerulosa is narrow and contains only scanty lipid. Fasciculata and reticularis contain abundant lipid. Frozen section. Oil Red O and mematoxylin. ×210.

in the interstitial tissue. In a few cases tubular damage was more severe and degenerative changes in the epithelium were noted (Fig. 3). Frank pelvic inflammation occurred in 7 rats; examples were seen in several stress groups but were absent from the control rats. Damage to the glomerular tuft was noted in rats stressed by repeated fracture, laparotomy, hemorrhage, or by burning (Table 2). The most extensive glomerular lesions occurred in a rat repeatedly burned, and at necropsy a blood pressure record of 162 mm. Hg was obtained (Fig. 4). Damaged glomeruli were shrunken and showed partial or complete adhesion to the capsule. In some the subcapsular space was enlarged and the parietal epithelium heightened. The normal morphology of such glomeruli was distorted further by an increase in structureless hyaline material within the tuft leading to obliteration of the capillary loops (Fig. 5). This damage was not associated with any abnormality of the renal arteries and proliferative arteriolosclerosis was not seen. Hypertensive vascular disease was absent from the other organs (pancreas, etc.) taken for examination.

Adrenals: Some adrenal hypertrophy occurred in the stressed rats (Table 2). The adrenals of the control rats showed a uniform pattern microscopi-

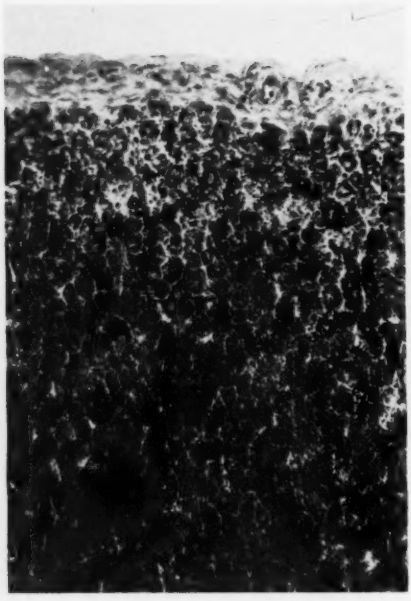


FIG. 7. Adrenal. Laparotomy. Zona glomerulosa is wider and is well filled with lipid. The cells of fasciculata are larger and the lipid droplets finer (cf. Fig. 6). Frozen section. Oil red O and H. ×210.

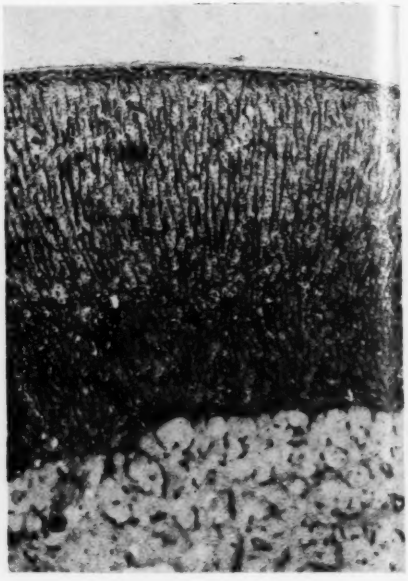


FIG. 8. Adrenal. Burn. Alkaline phosphatase activity is present in the cells and sinusoids of reticularis and adjacent fasciculata zones. It is confined to the sinusoids in zona glomerulosa and the medulla. Frozen section. Gomori's alkaline phosphatase method. ×50.

cally. Zona glomerulosa was narrow and composed of small cells with a homogeneous cytoplasm. Lipid depletion was virtually complete and the tests for cholesterol and birefringence were negative. Lipid globules, cholesterol deposits and birefringence were most intense in the outer two-thirds of the zona fasciculata and became less marked in the inner aspects of the cortex (Fig. 6).

The adrenal glands of the stressed rats presented a variable picture. The most consistent alteration from the control group was the enlargement of zona fasciculata and reticularis. Fine lipid droplets filled the cells throughout the zones and in some cases the reactions for cholesterol and birefringence were more intense. In some glands the appearance of the glomerulosa zone was identical with that seen in the control rats, while in others the width of zona glomerulosa was maintained and the cells contained lipid deposits, cholesterol and birefringent crystals (Fig. 7).

Some variations in the pattern of distribution of the phosphatases in the adrenal gland were noted. Alkaline phosphatase activity, as demonstrated by the method employed, was observed in the cells and sinusoids of zona fasciculata and reticularis of all rats, including controls. Similar locali-

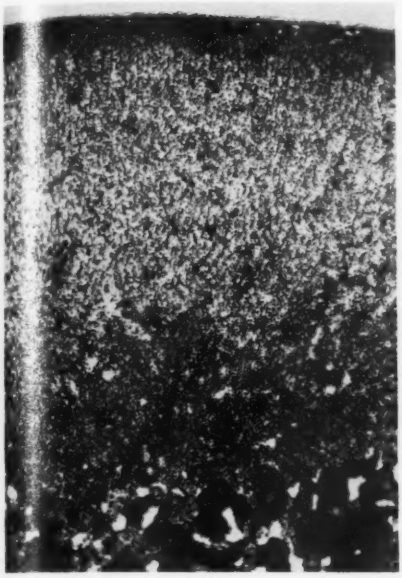


FIG. 9. Adrenal. Formalin injection. Acid phosphatase activity is most intense in zona glomerulosa and reticularis and in some of the medullary cells. Frozen section. Gomori's acid phosphatase method. ×50.

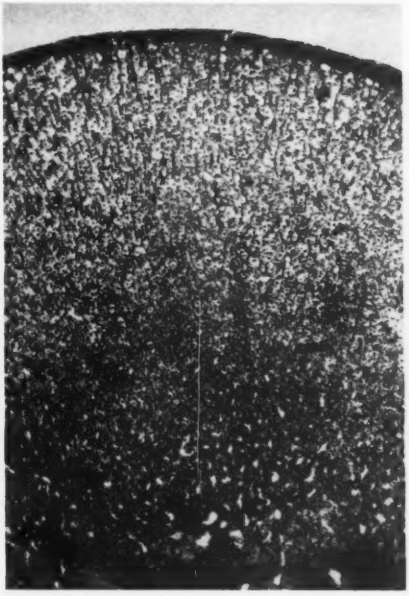


FIG. 10. Adrenal. Formalin injection. The pattern of acid phosphatase activity differs from Fig. 9 in that the zone of reaction in glomerulosa is narrowed. Frozen section. Gomori's acid phosphatase method. ×50.

zation was seen in zona glomerulosa of the adrenal in the majority of our groups, but in a few stressed animals glomerulosal activity was confined solely to the sinusoids (Fig. 8). Similarly an alteration in the pattern of acid phosphatase activity was most apparent in the zona glomerulosa and consisted of a variation in the width and intensity of the reaction (Figs. 9 and 10).

A clear correlation between the reactions of the adrenal gland, especially zona glomerulosa, and the incidence and severity of renal and myocardial damage could not be demonstrated.

Blood-pressure: Measurements were made in 4 groups of stressed rats and in the control group (Table 2) and were found to be significantly elevated in 3 rats. One burned rat had a mean pressure of 162, one rat subjected to limb ligation had a mean pressure of 149, and a second rat subjected to limb ligation had a mean pressure of 148 mm. Hg.

DISCUSSION

At least some of the stressors used in these experiments caused a greater amount of pathology in the kidney of some sensitized rats than occurred in

the control sensitized rats. The correlation between the ratings of gross renal damage and ratings of microscopic damage to the kidney was poor. In this and in earlier experiments it was found repeatedly that significant amounts of microscopic damage can occur in the blood vessels, glomeruli and tubules without visible signs of damage on the surface of the kidney. However, roughness, pitting, mottling and the presence of red spots on the surface of the kidney are always accompanied by significant microscopic changes.

The average amount of damage and the upper range of damage was less in the present experiments than previously seen in sensitized rats exposed to cold (3, 4). The accompanying increase in food intake was an important factor determining the extent of damage in the cold-exposed rats. In the present experiments the intake of the high salt diet was not increased above control values in the animals exposed to stress.

A careful search was made for gross and microscopic signs of other "diseases of adaptation." None were found. The stomach was free from ulcers, there were no swollen joints, rheumatic carditis, diabetes, tumors or any other pathologic changes outside the heart and remaining kidney.

These experiments yield no information on the role of the adrenal glands in the etiology of the damage. The average increase in adrenal weight was small in each experimental group. There was no clear correlation between an alteration in the histochemical pattern of the adrenal and the severity of renal and myocardial damage. The average weight of thymus was only moderately suppressed in the stressed animals. We are not aware of any data in the literature which have a direct bearing on the role of the adrenal glands in causing the pathology associated with exposure to non-specific stressors. It should be possible to compare stressed and non-stressed sensitized, adrenalectomized animals treated with a constant intake of adrenal cortical extract in order to test the hypothesis that a change in the secretory activity of the adrenal glands is the primary cause of the damage.

Some pathology occurs in the kidneys of some sensitized rats under non-stress conditions. The average amount of damage is increased in animals exposed to some stressors. The changes are much less impressive than can be caused by overdosing the sensitized rat with certain steroids or by exposure to cold. The basic conditions of these experiments (unilateral nephrectomy and a high load of sodium chloride) do not occur naturally. The stressors used were as severe as could be tolerated without causing death and they were imposed on the animal repeatedly. Whether or not these observations are interpreted to support or weaken the concept of Selye is a subjective matter. The present authors will maintain a cautious interest in the proposition that non-specific stress can cause diseases by derailment of these adaptive processes which are controlled by the adrenal cortex. Information is needed on the consequences of exposing normal animals to naturally occurring stressors and then sampling the changes which occur

throughout the entire life span. Such studies should yield information on the effect of amounts and kinds of stress upon longevity and the occurrence of diseases associated with ageing.

REFERENCES

1. SELYE, H. AND G. HEUSER. Fifth Annual Report on Stress. MD Publications, Inc. New York, 1955–1956.
2. SELYE, H: *Rev. Canad. Biol.* **2**: 501. 1943.
3. INGLE, D. J. AND B. L. BAKER: *Journ. of Lab. and Clin. Med.* **49**: 100. 1957.
4. CRANE, W. A. J., B. L. BAKER, AND D. J. INGLE: *Endocrinology* **62**: 2 and 216. 1958.
5. SELLERS, E. A. AND R. W. YOU: *Brit. Medical Journal* **4971**: 815. 1956.
6. INGLE, D. J.: *Amer. Journ. of Physiol.* **139**: 460. 1943.
7. PEARSE, A. G. E.: Histochemistry, Theoretical and Applied. Little, Brown and Company. Boston, 1954.
8. SYMINGTON, T.: *Brit. J. Exper. Path.* **32**: 58. 1951.

EFFECT OF GROWTH HORMONE AND THYROXINE ON BODY WEIGHT OF HYPOPHYSECTOMIZED C3H MICE[1]

ARDIS J. LOSTROH[2] AND CHOH HAO LI

Hormone Research Laboratory and the Department of Biochemistry, University of California, Berkeley, California

ABSTRACT

Bovine growth hormone was administered to hypophysectomized female C3H mice at increasing dose levels to maintain continuous growth. Within a period of 25 weeks the initial weight of the animals had doubled. Plateauing was observed at all dose levels, but it was found that gains could always be reinitiated by increasing the dose level. It was also demonstrated that although thyroxine was ineffective as an independent growth factor, when administered at a daily dose level of 0.0003 mg. together with growth hormone for longer than 8 weeks, it did act as a synergist with the latter in effecting body weight increases. When thyroxine in increasing dose levels was administered together with growth hormone, toxic effects were observed. 0.003 mg. of thyroxine plus growth hormone produced enlarged adrenal glands in all the mice so treated; from histological sections of the gland it was apparent that the enlargement could be attributed primarily to the extensive development of one region of the adrenal cortex, the juxtamedullary zone.

EXTENSIVE investigations in the rat have established that growth hormone from beef pituitary glands stimulates growth of the skeleton as well as increases in the body weight in that animal (1). Recently, it has been shown that the bovine hormone is also effective in stimulating skeletal growth in the hypophysectomized female mouse as measured by the quantitative response in the epiphyseal cartilage plate of the tibia when daily doses of growth hormone of from 1 to 20 μg. are administered over a period of 17 days (2). It was likewise demonstrated that when the growth hormone was injected for a period of only 4 days, as is routinely done in the rat assay, the mouse exhibits only a limited response; however, if thyroxine is injected along with the growth hormone, the sensitivity of the mouse to exogenous growth hormone can be increased approximately five-fold. As an extension of these studies, hypophysectomized female mice have been injected with growth hormone, thyroxine, or a combination of the two hor-

Received October 3, 1957.

[1] This work was supported in part by grants from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and from the Albert and Mary Lasker Foundation.

[2] Present address: Collège de France, Laboratoire d'Embryologie, 11 Place Marcelin Barthelot, Paris, France.

mc ues for a period of 25 weeks in order to ascertain the effect of the treatment on somatic growth as reflected in increases in body weight. The growth curves along with other pertinent data are presented herein.

METHODS

Female mice of the C3H strain were hypophysectomized at 35 days of age according to the technic described in an earlier publication (3). After the operation, 1 mg. of aureomycin plus 500 units of pencillin in 1 ml. of 5% glucose solution were given intraperitoneally. A similar injection was administered on the following day along with a subcutaneous injection of 0.25 mg. of hydrocortisone. Throughout the study, the animals were fed a modified McCollum's Diet I, *ad libitum* (4).

Administration of the hormone solution in single daily subcutaneous injections was initiated 12 days after hypophysectomy when the weights of the animals had fallen to a constant post-operative level. In most instances the studies extended over a period of 25 weeks; in the case of the thyroxine-treated groups, injections were discontinued at the end of 10 weeks, because a number of the hypophysectomized mice in these groups had died during the previous week and the survivors were in a poor condition. All animals were weighed every 7 days. At autopsy, the ovaries, uterus, adrenals, thymus and spleen were weighed on a Roller-Smith torsion balance; the adrenal glands were fixed in 10% neutral formol and, subsequently, frozen sections 10 μ in thickness were prepared and stained with oil red O or with Sudan black B. At this time the hypophyseal fossa was inspected with a binocular microscope, 12×, in order to establish that no pituitary remnants were visible in any of the operated mice.

The growth hormone used in this study was prepared from bovine pituitaries according to the procedure previously described (5). The DL-thyroxine was a Squibb product.

RESULTS

The growth curves obtained in the hypophysectomized mice with growth hormone alone, thyroxine alone, and the combination, are depicited in Figures 1 and 2. These groups each comprised a minimum of five animals; the following results were obtained:

I. *Hypophysectomized controls.* Following hypophysectomy, these mice lost 4 gm. and then maintained a constant body weight of approximately 15 gm. with only minor fluctuations throughout the ensuing 25-week period.

II. *Growth hormone in increasing doses.* Daily doses of from 5 to 500 μg. were administered during the course of the study. The hormone was given at an initial dose level of 5 μg. and in order that a steadily-rising growth curve could be maintained, the dosage was increased whenever the animals showed a gain of less than 1 gm. in a 7-day injection period. As shown in Table 1, the weights of the adrenals, ovaries, uterus, thymus and spleen were heavier than those of the non-treated control group; if the organ weights are expressed as functions of the body weight, there is no difference between the two groups.

III. *Growth hormone in a high dose.* Those mice receiving a daily dose of 0.5 mg. of growth hormone for 6 weeks doubled their initial weight within 3 weeks and thereafter maintained it at a constant level of 27 gm., a

weight comparable to that of the group given increasing dose levels of growth hormone over a period of 20 weeks. After these animals had attained a maximal weight, as indicated by no further increases for a period of 3 weeks, they were apportioned into two groups. Six mice received proportional levels of the original dose of growth hormone in order to ascertain the minimal level of hormone required to maintain their body weight; the remaining four mice were given no additional injections. Whereas a daily

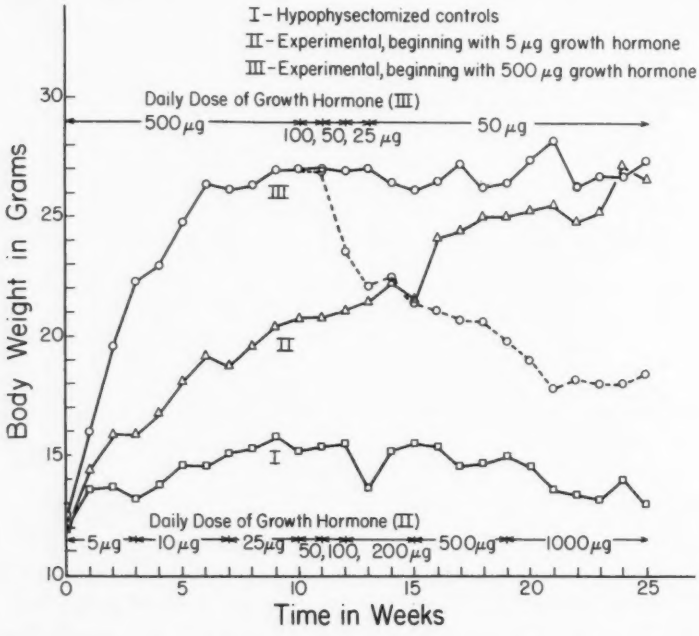


FIG. 1. Average body weight curves of female mice over a period of 25 weeks. All animals were hypophysectomized at 35 days of age and injections were begun 12 days later. Group I: Hypophysectomized control. Group II: Growth hormone in increasing doses. Group III: Growth hormone in a high dose (500 μg.) for 10 weeks followed by decreased dosages for maintaining the body weight.

dose of as low as 25 μg. was inadequate, 50 μg., or one-tenth of the original dose, was found to be sufficient to maintain the body weight during the ensuing 14 weeks. Withdrawal of the hormone led to an immediate loss of weight.

IV. *Thyroxine in a constant dose.* DL-thyroxine given at a dose level of 0.3 μg. was quite ineffective in promoting a sustained increase in body weight.

V. *Thyroxine in increasing doses.* The initial daily dose of thyroxine (0.3 μg) was increased in proportion to the levels of growth hormone that were administered to Group III. The results were similar to those obtained with a constant daily dose of 0.3 μg of thyroxine.

VI. *Growth hormone in increasing doses plus a constant daily dose of 0.3 μg of thyroxine.* Greater body weight gains were obtained with the combination of the two hormones than with growth hormone alone after the first 8 weeks of treatment, so that by the time of autopsy the former group weighed 33.0 gm. and the latter group, 26.7 gm. During the earlier period, their growth curves were parallel. Whereas the weights of the adrenals, ovaries and uterus of the group given the combination were comparable to

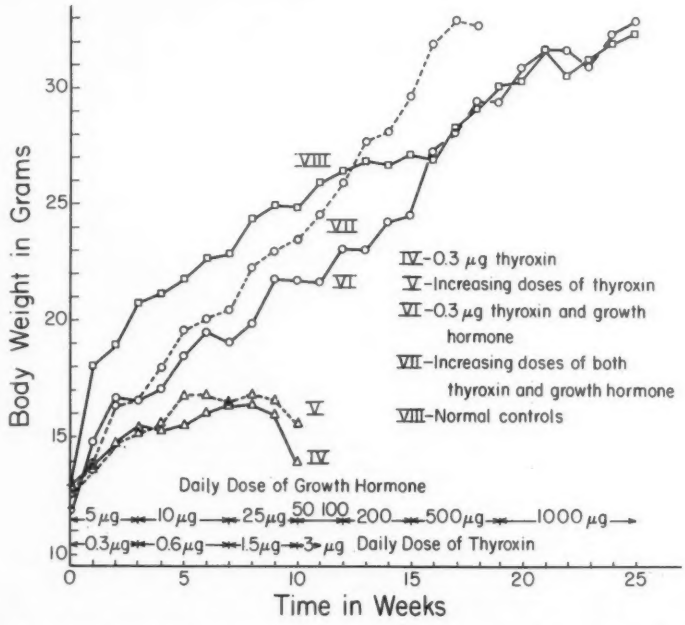

Body Weight in Grams
Time in Weeks
IV–0.3 μg thyroxin
V–Increasing doses of thyroxin
VI–0.3 μg thyroxin and growth hormone
VII–Increasing doses of both thyroxin and growth hormone
VIII–Normal controls
Daily Dose of Growth Hormone
5 μg 10 μg 25 μg 50 100 200 500 μg 1000 μg
0.3 μg 0.6 μg 1.5 μg 3 μg Daily Dose of Thyroxin

FIG. 2. Average body weight curves of female mice over a period of 25 weeks. Injected animals were hypophysectomized at 35 days of age and injections were begun 12 days later. Group IV: Thyroxine in a constant dose (0.3 μg.). Group V: Thyroxine in increasing doses. Group VI: Growth hormone in increasing doses plus thyroxine in a constant dose (0.3 μg.). Group VII: Growth hormone plus thyroxine, both in increasing doses. Group VIII: Intact control.

those of the growth hormone-treated group, the weights of the thymus and spleen were considerably greater (Table 1).

VII. *Growth hormone plus thyroxine, both in increasing doses.* The daily dose levels of the two hormones given to this group correspond to the doses administered separately to Groups III and V. After the 8th week, it was apparent that the general health of the animals was deteriorating progressively even though they continued to register weight gains; they were irritable, their coats became rough and their respiration was rapid. Dosages of thyroxine greater than 3 μg were not administered inasmuch as it seemed

unlikely that any of the hypophysectomized animals could tolerate higher levels of the hormone.

At autopsy it was found that these these animals had enlarged adrenals; the weights of the glands actually exceeded those of non-hypophysectomized control mice. Much of the observed increase in weight can be attributed to the proliferation of cells in the juxtamedullary region of the gland

TABLE 1. ORGAN WEIGHTS OF HYPOPHYSECTOMIZED* FEMALE C3H MICE INJECTED WITH GROWTH HORMONE, THYROXINE, OR A COMBINATION OF THE TWO HORMONES FOR A PERIOD OF 25 WEEKS

Treatment	Daily dose	No. of Animals	Body weight, gm.		Organ weights, mg.				
			Onset	Autopsy	Adrenal	Ovary	Uterus	Thymus	Spleen
	μg								
Hypophysectomized control (Group I)	0	5	12.0	13.0	1.46±0.1† (1.12)‡	0.63±0.3 (0.48)	4.1± 0.4 (3.2)	9.2±1.0 (7.1)	47±3 (36)
Growth hormone (Group II)	Incr.	6	12.0	26.5	2.63±0.2 (0.98)	1.37±0.1 (0.51)	7.3± 0.6 (2.7)	17.8±1.4 (6.6)	167±9 (63)
Growth hormone (Group III)	500×6 wks., 0×19 wks.	4	12.5	18.5	1.62±0.1 (0.88)	0.80±0.1 (0.43)	4.7± 0.3 (2.6)	4.6±0.6 (2.5)	61±1 (33)
Growth hormone (Group III)	500×6 wks., 50×19 wks.	6	12.5	27.5	2.22±0.2 (0.81)	1.23±0.1 (0.45)	6.5± 0.6 (2.4)	19.4±1.3 (7.1)	142±1 (52)
Thyroxine (Group IV)	0.3×10 wks.	6	13.0	15.5	1.34±0.1 (0.86)	0.62±0.1 (0.40)	5.1± 0.5 (3.3)	19.4±1.6 (12.5)	57±1 (37)
Thyroxine (Group V)	Incr. ×10 wks.	2	13.5	14.0	1.28 (0.91)			29.8 (21.3)	76 (54)
Growth hormone + Thyroxine (Group VI)	Incr. + 0.3	5	12.0	33.0	2.59±0.2 (0.78)	1.12±0.1 (0.34)	8.76±0.2 (2.7)	30.0±1.4 (9.1)	302±8 (92)
Growth hormone + Thyroxine (Group VII)	Incr. + Incr. ×20 wks.	6	12.5	33.0	7.9 ±0.9 (2.39)	0.90±0.1 (0.27)	10.2± 1.1 (3.1)	48.4±4.3 (14.7)	303±22 (92)
Normal control (Group VIII)	0	5	18.0	33.5	4.96±0.1 (1.47)	14.81±1.3 (4.41)	91.4±11.9 (27.2)	24.8±2.4 (7.3)	144±16 (43)

* All animals hypophysectomized at 35 days of age. Single daily subcutaneous injections begun at 47 days of age.
† Mean ± standard error.
‡ Values in parentheses represent organ weight in mg./10 gm. body weight.

(Fig. 3). In the mouse, a non-sudanophilic juxtamedullary zone is characteristic of the adrenal of the virgin female; this region was atrophic in the hypophysectomized control animals and was discernible but not conspicuous in the animals that had received growth hormone or growth hormone plus 0.3 μg. of thyroxine (Figs. 4 and 5). In frozen sections of the adrenal it was apparent that the cells of the enlarged zone contained varying, but on the whole substantial, amounts of cytoplasm, as well as sudanophilic droplets that stained with oil red O and with Sudan black B. At the same time, there was no suggestion that these adrenals were producing sex hormones at a level sufficient to stimulate increases in the weights of the uteri.

VIII. *Intact controls.* Over the period of 25 weeks, the body weight of these animals increased from 13 gm. to 32.5 gm. and small weight gains were still registered up to the time of autopsy at 220 days of age.

FIGS. 3–5. Frozen sections, 10 μ in thickness, of adrenal glands of female C3H mice hypophysectomized at 35 days of age. Sudan black B stain ×37.

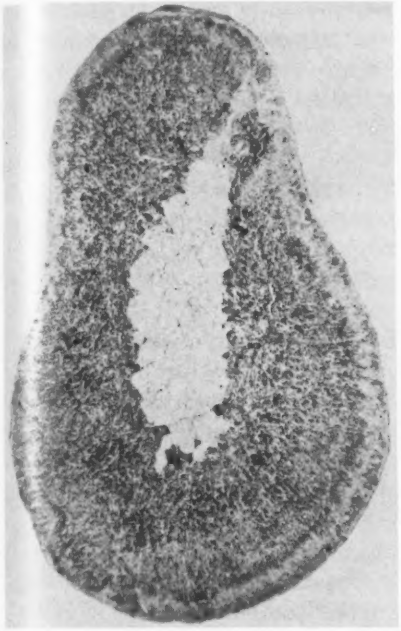


FIG. 3. Adrenal of mouse injected with increasing dosages of both growth hormone and thyroxine for a period of 20 weeks. Note the extensive development of the juxtamedullary region along with the appearance of sudanophilic material in this area.

FIG. 4. Adrenal of control, 25 weeks after hypophysectomy.

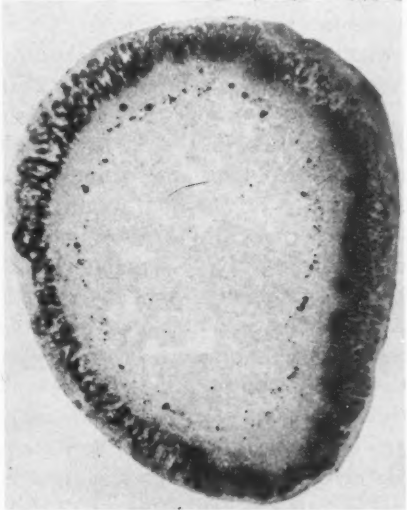


FIG. 5. Adrenal of mouse injected with increasing dosages of growth hormone plus 0.3 μg. of DL-thyroxine for a period of 25 weeks. The cells of the cortex exhibit less atrophy than do those of the control and a non-sudanophilic juxtamedullary zone is discernible.

DISCUSSION

Hypophysectomized female mice register body weight increases with daily doses of growth hormone as low as 5 μg. in short-term studies; how-

ever, if growth is to be maintained the dosage must be increased whenever the animals attain a new plateau or leveling off of the weight gain. This failure to exhibit continued growth, referred to hereafter as *plateauing*, was observed whether the initial dose was large or whether a small initial dose was increased intermittently over an extended period of time. A similar trend has been reported in the rat, although continuous growth has been obtained in hypophysectomized females of the Long-Evans strain for a period of as long as 437 days with daily dosages of purified growth hormone of from 0.1 to 0.4 mg. (7). In the mouse, on the other hand, an even higher dose level, 0.5 mg., was effective in promoting an increase in body weight for a period of 6 weeks only. Attempts to overcome growth stasis in the rat by administering extracts of rat pituitary (8) rather than extracts from other species, or by injecting adrenal corticoids in conjunction with bovine growth hormone (9) have been unsuccessful.

An extension of the studies in the plateaued mice that had been injected with 500 μg. of growth hormone for a period of 9 weeks revealed that a dose of 50 μg., or one-tenth that required to effect the increase in the body weight, could maintain that body weight (Fig. 1). Furthermore, the continued administration of a relatively low dose of growth hormone obviated any loss in body weight for many weeks whereas complete withdrawal resulted in an abrupt loss amounting to several grams followed by a gradual decline. It may be that a similar situation obtains in the adult animal; were this the case, comparatively large variations in the concentration of growth hormone available to the tissues could be tolerated without creating an imbalance in the physiological system. At the same time, a low level of the hormone could maintain the "status quo" of the system.

A second hormone that influences the growth rate of mice is thyroxine. Normal animals treated with thyroxine grow at a more rapid rate than do untreated controls and attain a maximal weight within a shorter period of time; however, the final weight of the treated group does not exceed that of the control (10, 11). Subsequent studies in hypophysectomized rats have demonstrated that thyroxine can effect slight weight gains for a period of approximately 40 days if the animals are allowed free access to food but no gains if their food intake is restricted to that of the controls (12). A similar response was evident in the hypophysectomized mouse. But, whereas thyroxine is quite unimpressive as an independent growth factor, its effectiveness as a synergistic agent with pituitiary growth hormone is readily demonstrable with respect to skeletal and body growth (2). The dose level of thyroxine does not appear to be a crucial factor so long as it is adequate but not toxic.

The adrenals, ovaries and uteri of the hypophysectomized mice treated with growth hormone were somewhat less atrophied than those of the controls; if the weights of these organs are expressed in terms of the body weight, the values for the two groups are comparable (Table 1). Similar

results with respect to the ovaries and uteri were obtained in the groups treated with growth hormone and thyroxine. On the other hand, the combination of the two hormones effected considerably greater increases in the splenic weights. As in the present study, enlargement of the spleen has been obtained in the rat following the administration of growth hormone (4). further studies are needed in order to determine if, in animals other than the female C3H mouse, thyroxine given along with growth hormone is more effective in stimulating splenic development than is growth hormone alone, and to ascertain the functional or biological significance of the enlargement of this organ under these conditions.

The adrenals of the hypophysectomized mice treated with increasing dose levels of both growth hormone and thyroxine were larger than those of any other group, including the normal control (Table 1). Although it is apparent from these results that the extensive development and disorganized expansion of the usually non-sudanophilic juxtamedullary region was confined to the group injected with increasing levels of both growth hormone and thyroxine, the significance of this development remains to be clarified. In the rat, the administration of growth hormone to either the intact or the hypophysectomized animal results in hypertrophy of the adrenal medulla (13), and in the intact animal the treatment may eventually give rise to a medullary tumor (14). Recently, it has been reported that the administration of 5 μg or 250 μg. of thyroxine plus growth hormone to intact female rats of the Sprague-Dawley strain causes considerable enlargement of the adrenal gland (15). The increase was due almost entirely to hypertrophy of the cortex, and no striking qualitative changes in the structure of the gland were observed. Whether or not the above-mentioned observations on the rat adrenal are in any way related to the unusual development of a peculiar zone in hypophysectomized mice treated with growth hormone plus toxic levels of thyroxine in the absence of their own pituitaries remains to be determined.

Acknowledgment

The authors wish to acknowledge the able technical assistance of Charles W. Jordan and Peggy Woodward.

REFERENCES

1. Li, C. H.: Harvey Lectures, Series XLVII, 181 (1950–51), 1952.
2. Lostroh, A. J. and C. H. Li: *Endocrinology* **60**: 308. 1957.
3. Lostroh, A. J. and C. W. Jordan, Jr.: *Proc. Soc. Exp. Biol. Med.* **90**: 267. 1955.
4. Li, C. H., P. Fønss-Bech, I. I. Geschwind, T. Hayashida, G. Hungerford, A. J. Lostroh, W. R. Lyons, H. D. Moon, W. O. Reinhardt and M. Sideman: *J. Exp. Med.* **105**: 335. 1957.
5. Li, C. H.: *J. Biol. Chem.* **211**: 555. 1954.
6. Howard-Miller, E.: *Am. J. Anat.* **40**: 251. 1927.
7. Simpson, M. E., H. M. Evans and C. H. Li: *Growth* **13**: 151. 1949.
8. Emerson, J. D. and G. M. Emerson: *Am. J. Physiol.* **182**: 521. 1955.

9. Emerson, J. D. and G. M. Emerson: *Endocrinology* **60:** 393. 1957.
10. Robertson, T. B.: *Austr. J. Exp. Biol. Med.* **5:** 69. 1928.
11. Koger, M., V. Hurst and C. W. Turner: *Endocrinology* **31:** 237. 1942.
12. Asling, C. W., M. E. Simpson, C. H. Li and H. M. Evans: *Anat. Rec.* **119:** 01. 1954.
13. Moon, H. D., M. E. Simpson, C. H. Li and H. M. Evans: Cancer Res. **11:** 35. 1951.
14. Moon, H. D., M. E. Simpson, C. H. Li and H. M. Evans: *Cancer Res.* **10:** 364. 1950.
15. Bois, P. and H. Selye: *J. Endocrin.* **15:** 171. 1957.

COMPARISON OF THE EFFECTS OF CORTISONE AND CERTAIN ANABOLIC-ANDROGENIC STEROIDS ON THE UPTAKE OF RADIOSULFUR IN A HEALING FRACTURED BONE

K. KOWALEWSKI

McEachern Cancer Research Laboratory and Department of Surgery, University of Alberta, Edmonton, Canada

ABSTRACT

The uptake of S^{35} in the fractured and intact humerus of male rats was determined and the ratio F/I of radioactivity of fractured (F) to intact (I) bone was calculated.

The effect of cortisone, 17-ethyl-19-nortestosterone and testosterone propionate on the F/I ratio was studied. Cortisone resulted in a significant decrease of F/I ratio as compared with normal control. Anabolic androgen 17-ethyl-19-nortestosterone exhibited a significant rise of S^{35} uptake in fractured bones and consequently high F/I ratios. Combined therapy with cortisone and 17-ethyl-19-nortestosterone offset the effect of cortisone on S^{35} uptake. Testosterone propionate had no significant effect.

It is apparent from this study that cortisone inhibits and 17-ethyl-19-nortestosterone promotes those processes of repair which may be measured by S^{35} uptake method, and that 17-ethyl-19-nortestosterone may protect connective tissue against the catabolic effect of cortisone.

IT HAS been demonstrated that cortisone exerts an inhibitory effect on the chemical and cellular processes associated with inflammation and repair (1). It is believed that this catabolic steroid may interfere with the elaboration or activation of mucopolysaccharides (2). Cortisone blocks the synthesis of chondroitin sulfate in embryonic and normal tissue, and arrests the growth of cartilage in embryo femur anlages (3, 4). This inhibitory effect of cortisone concerns both cellular elements of mesenchymal tissue and the ground substance.

It is apparent that the alterations of collagen fibrillogenesis provoked by cortisone and any other catabolic or anabolic hormone may be well studied by the radiosulfur uptake method (5). This method gives information regarding the location of the tissue sulfate. The selective deposition of radiosulfur in the cartilage, bones and healing wounds is now explained by the utilization of sulfate ions in the synthesis of chondroitin sulfate present in the connective tissue ground substance and in the collagen (6). Radiosulfur may be utilized for the study of the various stages of formation of collagen tissue during the process of healing and particularly for the study of normal and pathologic metabolism of cartilage and bone.

Received October 5, 1957.

In a previous report, a method for the study of the uptake of radiosulfur in the bones of rats was proposed (7). We were able to demonstrate that the factured humerus showed an increased uptake of S^{35} as compared with the uptake of the intact humerus of the same animal. This technique was used for the investigation of the anabolic effect of certain androgenic steroids (5, 8). It was shown that a synthetic anabolic steroid 17-ethyl-19-nortestosterone (9) when given to normal or castrated rats during the period of healing of fractures significantly stimulated the S^{35} uptake in fractured bones. It was interpreted that 17-ethyl-19-nortestosterone had marked effects on the synthesis of chondroitin sulfate in the collagen tissue. Testosterone propionate did not show such an activity under similar experimental conditions (8).

It is apparent from the above data that cortisone and 17-ethyl-19-nortestosterone have an opposite influence on those repair processes which may be studied by the S^{35} uptake method. The purpose of the present experiment was to compare the effects of cortisone, 17-ethyl-19-nortestosterone and testosterone propionate on the S^{35} uptake by healing fracture. An attempt was also made to offset the catabolic effect of cortisone by simultaneous administration of those androgenic steroids to the animals.

METHODS

Preparation of animals: Male rats of the Wistar strain having a weight range from 210 to 325 gm. and fed with commercial Miracle dog food were used for this study.

Under ether anesthesia a closed complete fracture of the right humerus was produced by grasping the foreleg between the fingers and the thumb of each hand and breaking the bone at its mid point. The animals were kept in separate cages, and all made a satisfactory recovery from anesthesia. The fractures were left to heal unsupported. Three weeks following the fracture the rats were sacrificed.

Radiosulfur (S^{35} in H_2SO_4, Atomic Energy, Canada) was given in doses of 200 microcuries by intraperitoneal injection 24 hours prior to sacrifice. The dose was dissolved in 2 cc. of distilled water together with 8 mg. of sodium sulfate (7).

Treatment: The hormones were given by injection or per os during the three week period of healing of the fracture, 4 doses the first week and 3 doses weekly for the next two weeks. Following doses were used:

Cortisone (Merck), 10 mg. in 0.5 cc. of saline, subcutaneously;

17-ethyl-19-nortestosterone (Searle & Co.), 5 mg. in 0.4 cc. of sesame oil, intramuscularly;

17-ethyl-19-nortestosterone, 5 mg. per os, crushed tablet, mixed with food;

Testosterone propionate (Gen. Bioch. Inc.), 5 mg. in 0.4 cc. of sesame oil, intramuscularly.

Animals were divided into the following seven groups:

1—no treatment; 2—given 17-ethyl-19-nortestosterone in oil;
3—given testosterone propionate; 4—given cortisone;
5—treated with cortisone and 17-ethyl-19-nortestosterone in oil;
6—treated with cortisone and 17-ethyl-19-nortestosterone per os;
7—given cortisone and testosterone propionate.

Measurement of radiosulfur: After sacrifice of the rats the fractured normal humeri were removed, cleaned and dried at room temperature for 24 hours, following which their weights were recorded.

The bones were then dissolved by the nitric-perchloric acid wet-digestion procedure described in detail in our previous report (7).

The final precipitate of radioactive barium sulfate was transferred to planchettes and the counting carried out using a thin mica window Tracerlab G-M tube. A Tracerlab Superscaler S.C. 18A was used for recording.

The results were calculated in counts per minute (c.p.m.) per 100 mg. of tissue and presented as F/I ratio of radioactivity. This is the ratio of the uptake (c.p.m./100 mg.

TABLE 1. WEIGHT OF FRACTURED (W.F.) AND INTACT (W.I.) HUMERI EXPRESSED AS W.F./W.I. RATIO, THREE WEEKS AFTER FRACTURE

Experimental group	No. of rats	Values	W.F./W.I.
1	30	Mean	1.23
		S.D.	±0.10
		Range	1.11–1.39
2	20	Mean	1.30
		S.D.	±0.19
		Range	1.03–1.89
3	20	Mean	1.30
		S.D.	±0.18
		Range	0.93–1.62
4	25	Mean	1.27
		S.D.	±0.11
		Range	1.07–1.48
5	20	Mean	1.18
		S.D.	±0.12
		Range	0.99–1.90
6	20	Mean	1.27
		S.D.	±0.14
		Range	1.03–1.55
7	18	Mean	1.30
		S.D.	±0.11
		Range	1.13–1.63

of bone)-of S^{35} by the fractured humerus (F) to the uptake of S^{35} by the corresponding intact bone (I). The data obtained from the study of normal untreated rats and from the animals given 17-ethyl-19-nortestosterone and testosterone propionate were in part included in our previous reports (5, 8).

RESULTS

The results are summarized in the accompanying tables. The difference in weight between fractured (W.F.) and intact bone (W.I.) was expressed as W.F./W.I. ratio, and is given in Table 1. It may be seen that this ratio is not significantly affected by any treatment used in this study. This observation excludes the possibility that the data concerning the S^{35} uptake might be influenced by the change of W.F./W.I. ratio due to a treatment.

Table 2 presents the data on the study of radioactivity in fractured and intact humeri. There is a significant increase of F/I ratio in animals treated with 17-ethyl-19-nortestosterone and an equally significant reduc-

TABLE 2. RATIO F/I OF THE RADIOACTIVITY IN FRACTURED (F) AND INTACT (I) HUMERI, THREE WEEKS AFTER FRACTURE AND 24 HRS. AFTER THE ADMINISTRATION OF S^{35}

E.N.T. = 17-ethyl-19-nortestosterone
T.P. = testosterone propionate

Group	Condition	No. of rats	Values	F/I
1	No treatment	30	Mean S.D. Range	2.11 ±0.30 1.61–2.80
2	Injected with 50 mg. of E.N.T.	20	Mean S.D. Range	4.38 ±1.10 3.05–7.69
3	Injected with 50 mg. of T.P.	20	Mean S.D. Range	1.70 ±0.46 0.87–2.76
4	Injected with 100 mg. of cortisone	25	Mean S.D. Range	0.70 ±0.20 0.40–0.99
5	Injected with 100 mg. of cortisone and 50 mg. of E.N.T.	20	Mean S.D. Range	1.60 ±0.31 1.09–2.11
6	Injected with 100 mg. of cortisone and given 50 mg. of E.N.T. per os.	20	Mean S.D. Range	2.14 ±0.59 1.54–3.57
7	Injected with 100 mg. of cortisone and 50 mg. of T.P.	18	Mean S.D. Range	1.14 ±0.24 0.58–1.45

tion of this ratio in rats given cortisone, as compared with normal controls. Testosterone propionate does not improve the F/I ratio in the similar experimental conditions.

It may be noted that the simultaneous treatment with cortisone and 17-ethyl-19-nortestosterone partially offset the effect of cortisone, and this protective action is very significant in the group given 17-ethyl-19-nortestosterone per os. The effect of testosterone propionate on F/I ratio in cortisone treated rats, cannot be considered as significant.

DISCUSSION

The inhibitory effect of cortisone on tissue repair as demonstrated by the other techniques, was also proved in our experimental conditions. Because cortisone blocks the production of mucopolysaccharides which specifically incorporate labelled sulfate, the proposed uptake method seems adequate to study catabolic action of this hormone.

This method also shows that the anabolic steroid 17-ethyl-19-nortestosterone stimulates bones healing. This probably means that 17-ethyl-19-nortestosterone promotes the synthesis of collagen during the process of repair. Combined therapy protects connective tissue against the effect of cortisone. 17-ethyl-19-nortestosterone given by injection is less effective

than given per os. Under described experimental conditions the injection of 7-ethyl-19-nortestosterone in oil occasionally resulted in local infection and this factor may be responsible for the results. 17-ethyl-19-nortestosterone per os was well tolerated, and in combination with cortisone assured very significant protection of animals against the catabolic effect of cortisone, as determined by S^{35} uptake method.

Testosterone, which is considered to be a protein anabolic hormone (10), and is clinically used as a factor promoting bone growth (11), did not show such an effect on the repair of fractured bones in this study. It also did not demonstrate any significant protection in rats treated with cortisone.

The practical value of this finding for clinical use is difficult to foresee. However, the possibility of offsetting the catabolic effects of cortisone by some new synthetic anabolic steroids shall be seriously considered.

Acknowledgments

This project was supported in part by a research grant from G. D. Searle & Co., Chicago, Illinois, U. S. A. who also supplied us with 17-ethyl-19-nortestosterone (Nilevar).

Mrs. B. Rhind and Mr. P. Carson are thanked for technical assistance.

REFERENCES

1. Cavallero, C.: *Rev. Canad. de Biol.* **12**: 189. 1955.
2. Asboe-Hansen, G.: Connective Tissues. Trans. V-th Conference, J. Macy Jr. Found. 1954. p. 123.
3. Layton, L. L.: *Proc. Soc. Exper. Biol. and Med.* **76**: 596. 1951.
4. Buno, W. and H. Goyena: *Proc. Soc. Exper. Biol. and Med.* **89**: 622. 1955.
5. Kowalewski, K. and F. Gouws: *Surg. Gyn. & Obst.* **105**: 1. 1957.
6. Dziewiatkowski, D. D.: *J. Biol. Chem.* **189**: 187. 1951.
7. Osborne, J. and K. Kowalewski: *Surg. Gyn. & Obst.* **103**: 38. 1956.
8. Kowalewski, K. and R. T. Morrison: *Can. J. Bioch. Physiol.* **35**: 771. 1957.
9. Saunders, F. J. and V. A. Drill: *Endocrinology* **58**: 567. 1956.
10. Kochakian, C. D. and J. Dolphin: *Am. J. Physiol.* **180**: 317. 1956.
11. Sobel, E. H., Raymond, S., Quinn, K. V. and N. B. Talbot: *J. Clin. Endocrinol. & Metab.* **16**: 241. 1956.

CHANGES IN THE ADRENAL OF THE HYPOPHYSECTOMIZED C3H MOUSE WITH α-CORTICOTROPIN AND GROWTH HORMONE[1]

ARDIS J. LOSTROH[2] AND PEGGY WOODWARD

Hormone Research Laboratory, University of California, Berkeley, California

ABSTRACT

Changes in the adrenal gland of the C3H mouse and in the distribution of lipid therein as a consequence of hypophysectomy were investigated, and replacement therapy with α-corticotropin and growth hormone, alone or in combination, was instituted. 20 days after removal of the pituitary, the adrenal cortex manifested an atrophy similar to that described for other strains of mice and for the rat. α-Corticotropin at daily dose levels of 0.020 mg. and 0.050 mg. given for 7 days restored the *zona fasciculata* of the cortex and increased the histologically-detectable lipid in the *zona glomerulosa*. A dose of 0.10 mg. increased the weight of the adrenals by 100 per cent and effected a substantial depletion of sudanophilic lipid from the cortex. After treatment with growth hormone, the adrenal glands were not significantly heavier than those of the untreated hypophysectomized controls. On the other hand, the adrenals from animals injected with ACTH plus growth hormone were substantially heavier than the glands of animals injected with the same dosage of ACTH alone. These results are interpreted as indicating that the concurrent administration of growth hormone enhances the effectiveness of ACTH.

SINCE the isolation of the pure adrenocorticotropic (ACTH) peptide (1, 2, 3), many of the biological activities originally ascribed to the protein hormone have been re-investigated. Studies conducted in human patients have demonstrated the efficacy of the peptide in stimulating the release of adrenocorticoids (4). Recently, a thorough evaluation of the biological properties of α-corticotropin in the Long-Evans rat, including direct and indirect effects, has been published (5). No information has been reported with respect to the direct effects of α-corticotropin on the adrenal gland of the mouse, although it has been demonstrated that the hormone is a potent agent for mobilizing lipid to the liver of this animal after fasting (5). In the present investigation, particular attention has been given to the changes observed in the weight of the adrenal and in the distribu-

Received October 5, 1957.

[1] This work was supported in part by grants from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and from the National Institutes of Health, United States Public Health Service (No. G-2907).

[2] Present address: Collège de France, Laboratoire d'Embryologie, 11 Place Marcelin Barthelot, Paris, France.

tion of lipid in the zones of the gland as a consequence of hypophysectomy and subsequent replacement therapy with α-corticotropin or growth hormone, or with combinations of these two hormones.

EXPERIMENTAL PROCEDURE

Female mice of the C3H strain were hypophysectomized at 35 days of age according to the technique described in an earlier publication (6); single daily subcutaneous injections were begun on the 13th day after operation and were usually continued for 7 days. The only exception to this regimen was introduced in the case of growth hormone given together with ACTH, since the hypophysectomized mice rarely survived for longer than 2 hours following the injection of this combination.[3] This experimental group was pretreated with growth hormone for one week, beginning 12 days after hypophysectomy, and was then injected simultaneously with both hormones. For an appropriate control, the animals that received only growth hormone were likewise given a total of 14 rather than 7 injections. At autopsy, the two adrenal glands were dissected free from surrounding tissue, weighed, and immediately fixed in 10% neutral formalin. Subsequently, the glands were infiltrated with gelatin, and frozen sections, 10 μ in thickness, were prepared and stained with oil red O or with Sudan black B.

The ACTH-F fraction was obtained from an acid-acetone powder derived from sheep glands and the α-corticotropin was obtained by further purification of this material (1); both were administered as solutions in 16% gelatin. The growth hormone, isolated from bovine pituitaries (7), was found to contain no detectable ACTH activity in the ascorbic acid-depleting assay, and 0.04 U.S.P. units of thyrotropin (TSH) per mg.[4] The DL-thyroxine was a Squibb product.

RESULTS

Hypophysectomy. C3H female mice hypophysectomized at 35 days of age showed substantial atrophy of the adrenal glands 20 days after the operation. The weight of the paired glands was found to have decreased from 3.7 mg. to 1.9 mg. (Table 1), and the adrenal cortex manifested histologically detectable changes, which indicate that it is dependent upon the pituitary for the maintenance of a normal appearance. The *zona fasciculata* was shrunken, the individual fascicular cells showed marked atrophy, and the *zona glomerulosa* had come to occupy about one-fourth of the cortex. Although no subglomerular sudanophobic zone was visible in the mouse adrenal, the cells of the glomerulosa could generally be distinguished from those of the fasciculata since the former are arranged in irregularly shaped nests between which the blood channels pass. The lipid in the *zona glomerulosa* was finely dispersed and sparse, but the concentration of the material that could be stained with either oil red O or Sudan black B did not seem

[3] The rapidity with which the typical symptoms such as lethargy and irritability developed following the injection of the two hormones suggested that changes in the blood glucose level might be associated with their appearance. No investigation of blood glucose levels in the cachetic animals was undertaken. However, in the limited number of instances in which determinations were made values in the diabetic range, 2–3 times those of the hypophysectomized controls, were observed.

[4] We wish to thank Dr. R. W. Bates of the National Institute of Arthritis and Metabolic Diseases for the assay of TSH contamination in the growth hormone preparation.

to be reduced as a consequence of hypophysectomy (Figs. 1A and 1B). I the *zona fasciculata*, the cells retained their typical cord-like arrangement but they did show some depletion of sudanophilic material, as illustrated in Figure 1B, where it can be seen that the staining reaction is less intense in the fasciculata of the hypophysectomized animal than in that of the normal control.

Treatment with ACTH. Both pure α-corticotropin and the partially purified ACTH promoted weight increases in the adrenal glands of the hypophysectomized mice. A daily dose of approximately 0.050 mg. of the pure

TABLE 1. EFFECT OF HYPOPHYSECTOMY* AND OF ACTH ON THE WEIGHTS OF THE ADRENALS AND THYMUS GLANDS OF THE C3H MOUSE

Treatment†	Daily Dose, mg.	No. of mice	Body weights, gm.			Organ weights, mg.	
			Day of operation	Day of 1st injection	At autopsy	Adrenals	Thymus
Normal	0	6	13.5		18.5	3.7 ± 0.1‡	43 ± 3
Hyphysectomy	0	5	15	12	12	1.9 ± 0.1	22 ± 4
ACTH-F	0.120	8	15	12	12.5	4.1 ± 0.3	—
ACTH-F	0.200	14	15.5	12.5	13	5.1 ± 0.2	—
ACTH-F	0.375	6	14	11.5	13	5.4 ± 0.3	—
α-Corticotropin	0.020	5	18	13	14.5	3.0 ± 0.2	8.4 ± 0.6
α-Corticotropin	0.050	4	17	13.5	15.5	4.2 ± 0.2	—
α-Corticotropin	0.100	4	18.5	13	14.5	4.1 ± 0.3	—

* Female mice hypophysectomized at 35 days of age and sacrificed at 54 days.
† Single daily subcutaneous injections begun at 47 days and continued for 7 days.
‡ Mean ± standard error.

peptide was required to produce adrenal weights comparable to those of the non-hypophysectomized control animals (Table 1). Involution of the thymus was associated with the adrenal stimulation. From the histological sections it was evident that both the *zona glomerulosa* and the *zona fasciculata* had been affected by the ACTH (Figs. 1C and 1D). The lipid distribution was found to be uniform throughout the two zones, the individual cells were large, and the nuclei had a normal appearance.

As would be expected on the basis of the results obtained in the rat (5, 8), dose levels of 0.10 mg. of α-corticotropin and 0.2 mg. of ACTH-F induced hypertrophy of the gland accompanied by an extensive depletion of the histologically-detectable lipid. With this degree of hypertrophy of the fascicular cells, the non-sudanophilic juxtamedullary zone[5] that is characteristic of the adrenal gland of the virgin female mouse (11), and that was discernible in all of the other groups studied, in this instance could not be distinguished as a region distinct from the inner fasciculata when examined in frozen sections stained with Sudan black B. Vacuolation of the cortical cells, particularly those of the outer fasciculata, also

[5] The juxtamedullary zone probably corresponds to the highly developed reticular zone described by Howard-Miller (9) and studied in some detail by Jones (10).

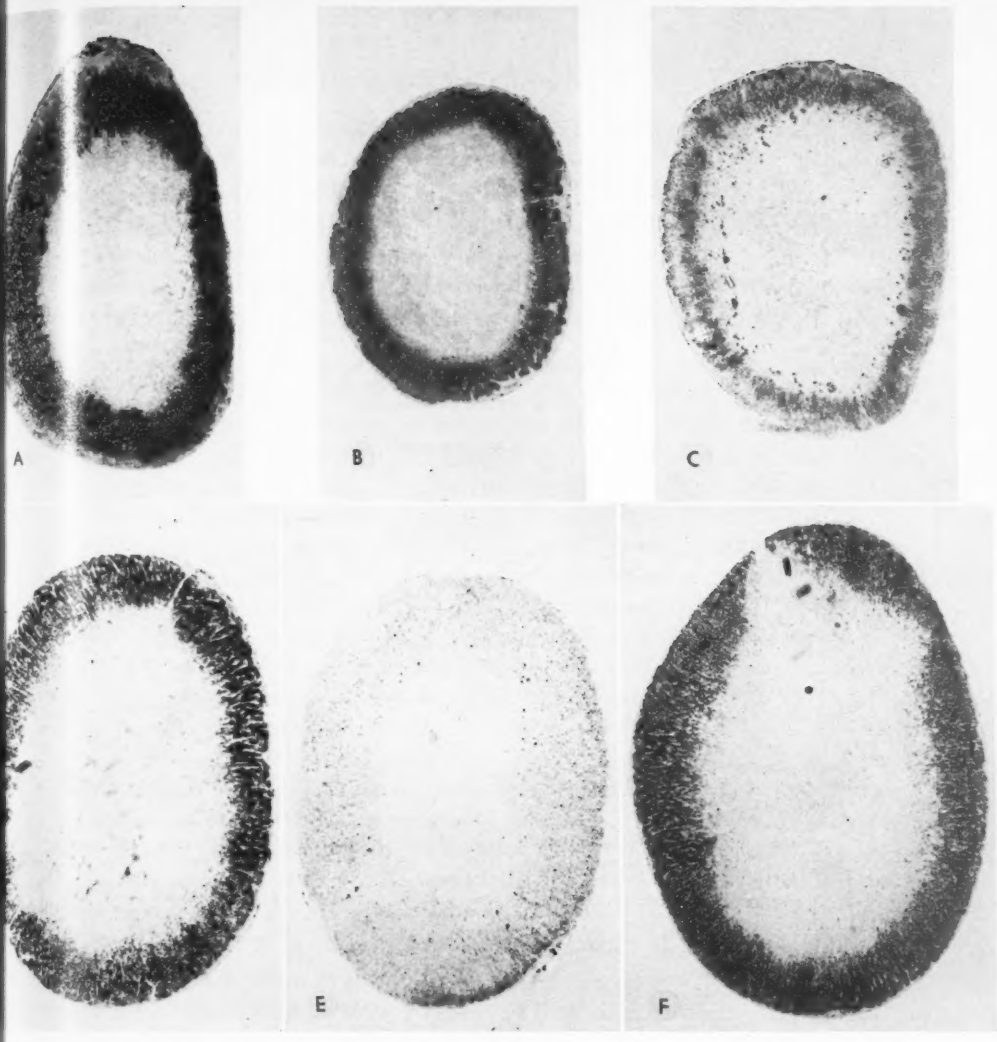


PLATE I.

FIG. 1. Frozen sections of adrenal glands of female C3H mice, 10 micra in thickness. Sudan black B stain, ×37.

A. Adrenal of normal mouse, 54 days of age. The *zona glomerulosa* contains a moderate amount of finely-disbursed lipid and the *zona fasciculata* is laden with lipid.

B. Adrenal of mouse, hypophysectomized at 35 days of age, 20 days after the operation.

C. Adrenal of hypophysectomized mouse injected with 0.050 mg. of α-corticotropin for 7 days. The *zonae glomerulosa* and *fasciculata* contain considerable lipid and the cortical cells are large.

D. Adrenal of hypophysectomized mouse injected with 0.10 mg. of α-corticotropin for 7 days. The *zona glomerulosa* contains some lipid; the cells of the *zona fasciculata* are hypertrophied and nearly depleted of sudan-staining material.

E. Adrenal of hypophysectomized mouse injected with 0.10 mg. of growth hormone for 14 days.

F. Adrenal of hypophysectomized mouse injected with 0.10 mg. of growth hormone for 7 days and with a combination of 0.10 mg. of growth hormone and 0.050 mg. of α-corticotropin for 7 days.

occurred. These cells were greatly hypertrophied and contained large vesicular nuclei.

Treatment with ACTH and Growth Hormone. The weights of the adrenal glands from hypophysectomized mice injected with a daily dose level of 0.10 mg. of growth hormone were not significantly higher than those of the glands from hypophysectomized controls, although the cells throughout the cortex of the treated animals appeared to be somewhat less atrophic

TABLE 2. EFFECT OF ACTH, GROWTH HORMONE AND THYROXINE, ALONE AND IN COMBINATION, ON THE WEIGHTS OF THE ADRENALS AND THYMUS OF THE HYPOPHYSECTOMIZED C3H MOUSE*

Treatment	Daily dose mg.	No. of mice	Body weights, gm.			Organ weights, mg.	
			Day of operation	Day of 1st injection	At autopsy	Adrenals	Thymus
Saline	0	5	15	12	12	1.9 ± 0.1†	22 ± 4
ACTH-F‡	0.200	14	15.5	12.5	13	5.1 ± 0.2	—
α-Corticotropin‡	0.050	4	17	13.5	15.5	4.2 ± 0.2	—
Growth Hormone§	0.100	10	18	12.5	19	2.2 ± 0.2	33 ± 3
DL-Thyroxine‡	0.0003	3	16	12	13	2.1	26.5
ACTH-F + Growth Hormone	0.200 + 0.100	6	16.5	13	16.5	7.0 ± 0.3	—
ACTH-F + DL-Thyroxine	0.200 + 0.0003	9	17	13	14	4.8 ± 0.2	—
α-Corticotropin + Growth Hormone	0.050 + 0.100	7	16	12	17.5	5.1 ± 0.2	7 ± 0.3

* All mice hypophysectomized at 35 days of age and sacrificed at either 54 or 61 days
† Mean ± standard error.
‡ Single daily subcutaneous injections begun at 47 days and continued for 7 days.
§ Single daily subcutaneous injections begun at 47 days and continued for 14 days.

and in several instances the staining reaction with Sudan black B was less intense (Fig. 1E). On the other hand, the weights of the adrenal glands from the animals injected with ACTH plus GH were substantially higher than the weights of the glands from animals injected with the same dosage of ACTH alone. For example, the adrenals of mice injected with 0.050 mg. of α-corticotropin weighed 4.2 mg.; those of mice given the same dose of α-corticotropin plus 0.10 mg. of growth hormone weighed 5.1 mg. (Table 2). At the same time, there was no indication that growth hormone given in conjunction with α-corticotropin effects any changes that cannot be produced with higher dose levels of α-corticotropin itself (Fig. 1F).

Treatment with ACTH and Thyroxine. The growth hormone preparation used in this investigation did contain 0.04 U. S. P. units of TSH/mg. That this level of contamination could be responsible for the synergism obtained when growth hormone is administered along with ACTH seems unlikely. However, in order to establish that the small amounts of thyroid hormone that might be released under the influence of a TSH contaminant could

not duplicate the effects on the adrenal gland observed when growth hormone is injected in conjunction with ACTH, dose levels of 0.0003 mg. of DL-thyroxine and 0.20 mg. of ACTH were administered to one group of the hypophysectomized mice. It can be seen in Table 2 that the weights of the adrenal glands from animals given the combination treatment were no greater than those from animals given ACTH alone.

DISCUSSION

The administration of α-corticotropin to hypophysectomized female mice was found to promote weight increases in the adrenal gland and to effect a redistribution of lipid in the cortex. The weight of the adrenals was restored to that of the normal control with a daily dose level of 0.050 mg. At this level, the intensity of the lipid stain was also similar to that seen in the normal animal and substantial amounts of finely disbursed droplets were present in the cells of the *zonae glomerulosa* and *fasciculata*. Although it has been amply demonstrated that the inner zones of the adrenal cortex undergo atrophy following hypophysectomy (12) and that in the rat this atrophy can be fully repaired with pure ACTH (5), there is disagreement as to whether or not the *zona glomerulosa* is in any way dependent upon the pituitary for its maintenance and function. It seems clear that the width of the *zona glomerulosa* in either the rat or the mouse is little if at all reduced for some time after hypophysectomy and that the individual glomerular cells do not exhibit a cellular shrinkage comparable to that of the cells of fasciculata (13, 14, 15). Nevertheless, changes in lipid distribution following the administration of ACTH have been demonstrated in both the rat (5, 16) and the mouse (17), and these earlier observations have been confirmed by the present study. Whether or not a redistribution of lipid is accepted as an indication of cellular activity, it can still be said that the *zona glomerulosa* of the hypophysectomized mouse does manifest histologically detectable alterations following the administration of pure α-corticotropin.

Considerable enlargement of the adrenal glands can be produced by treatments with ACTH; in the hypophysectomized rat, such abnormal glands usually contain little subdanophilic material (8). A similar situation obtains in the mouse. Although one might expect that if α-corticotropin increases the amount of sudanophilic material in the adrenal gland at a dose level of 0.050 mg., an increase would also be effected at a level of 0.10 mg. Nonetheless, the fact that, on the contrary, a decrease in sudanophilic material is observed does not necessarily indicate a decrease in the activity of the gland. It should be recognized that the intensity of the reaction obtained with a given staining agent is at best an indication of the quantity of reactive material that is present in the tissue under the arbitrary conditions chosen for the study. Thus, in the case of the adrenal it provides no

direct information as to the activity of the gland in terms of the synthesis or release of the steroid products, information that can only be implied from other histological criteria such as cell size, nuclear detail and vascularity, or derived from direct measurements of the corticoid output from the glands (18).

As discussed above, α-corticotropin influenced the lipid distribution in the *zona glomerulosa* and the *zona fasciculata* of the mouse adrenal and promoted considerable increase in the weights of the glands. The combination of α-corticotropin and growth hormone effected a similar response, but the weights of the adrenal glands from animals given both hormones were consistently heavier than those given only the α-corticotropin. Growth hormone itself caused a slight increase in the weight of the adrenal; nonetheless, the increase observed in the weight of the adrenals of animals treated with growth hormone alone over the weight of the adrenals of hypophysectomized controls should represent the maximal contribution that the stimulus afforded by growth hormone can make to the total weight of the gland. This amounts to only 20% of the increase elicited by growth hormone plus 0.050 mg. of α-corticotropin, and 10% of the increase elicited by growth hormone plus 0.20 mg. of the ACTH-F fraction. Clearly, the effectiveness of growth hormone in promoting weight increases in the adrenal gland of the hypophysectomized C3H mouse is related to the dosage of ACTH that is administered in conjunction with it. Within the dose range studied, the synergistic action exercised by growth hormone became more pronounced as the dose of ACTH was increased. Inasmuch as these results parallel the results previously obtained in the hypophysectomized rat (8), further support is given to the contention that the effect of growth hormone on the adrenal is distinct from the effect of ACTH and that growth hormone actually enhances the action of the latter.

Acknowledgment

The authors wish to express their appreciation to Dr. C. H. Li for his active interest in the project and for the pituitary hormone preparations used in this study. They also wish to thank Dr. W. R. Lyons for his criticism of the manuscript. The study was facilitated by the able technical assistance of C. W. Jordon.

REFERENCES

1. Li, C. H., I. I. Geschwind, J. S. Dixon, A. L. Levy and J. I. Harris: *J. Biol. Chem.* **213**: 171. 1955.
2. White, W. F.: *J. Am. Chem. Soc.* **75**: 503. 1953.
3. Bell, P. H.: *J. Am. Chem. Soc.* **76**: 5565. 1954.
4. DiRaimondo, V. and D. Island: *J. Clin. Endocrinol. & Metab.* **15**: 856. 1955.
5. Li, C. H., P. Fønss-Bech, I. I. Geschwind, T. Hayashida, G. Hungerford, A. J. Lostroh, W. R. Lyons, H. D. Moon, W. O. Reinhardt and M. Sidema. *J. Exp. Med.* **105**: 335. 1957.
6. Lostroh, A. J. and C. Jordan, Jr.: *Proc. Soc. Exp. Biol. & Med.* **90**: 267. 1955.
7. Li, C. H.: *J. Biol. Chem.* **211**: 555. 1954.

9. Lostroh, A. J. and C. H. Li: *Acta Endocrinologica* **25:** 1. 1957.
10. Howard-Miller, E.: *Am. J. Anat.* **40:** 251. 1927.
11. Jones, I. C.: *Endocrinology* **44:** 427. 1949.
12. Moog, F., C. J. Bennett, and C. M. Dean, Jr.: *Anat. Rec.* **120:** 873. 1954.
13. Smith, P. E.: *Am. J. Anat.* **45:** 205. 1930.
14. Deane, H. W. and R. O. Greep: *Am. J. Anat.* **79:** 117. 1946.
15. Lever, J. D.: *Endocrinology* **58:** 163. 1956.
16. Leblond, C. P. and W. O. Nelson: *Bull. d'histol. appl. à la Physiol. et à la Pathol.* **14:** 181. 1937.
17. Wexler, B. C., A. P. Rinfret, A. C. Griffin and H. L. Richardson: *Endocrinology* **56:** 120. 1955.
18. Jones, I. C.: *Endocrinology* **45:** 514. 1949.
19. Hofmann, F. G.: *Endocrinology* **59:** 712. 1956.

STEROIDOGENIC PROPERTIES OF PURIFIED CORTICOTROPINS[1]

GORDON L. FARRELL, RONALD B. FLEMING, ELIZABETH W. RAUSCHKOLB, FRANK M. YATSU, MICHAEL McCALLY AND CHARLES H. ANDERSON

Department of Physiology, Western Reserve University School of Medicine, Cleveland, Ohio

ABSTRACT

δ_1 and β corticotropins, prepared by Bell and co-workers, were assayed for hydrocortisone and aldosterone stimulating activity in the decerebrate dog. The two preparations were found to be of approximately equal potency in stimulating hydrocortisone secretion. However, δ_1 corticotropin proved to be several times as active as β corticotropin in stimulating the secretion of aldosterone. The aldosterone stimulating activity of δ_1 corticotropin is destroyed by heating in alkali, indicating that it is not due to the melanocyte stimulating activity known to be present in this preparation.

NUMEROUS, frequently conflicting, reports of the aldosterone stimulating activity of ACTH have appeared in the literature. In the hypophysectomized dog a modest increase in the rate of secretion of the steroid has been reported to follow the infusion of ACTH. The increase was small when compared to the effect of ACTH on the secretion of the other corticosteroids (1). In the rat, however, a much larger increase in aldosterone secretion after ACTH has been demonstrated (2). The *in vitro* effect of ACTH on aldosterone synthesis was found to be inconsistent and to vary with the preparation studied (3). In man, the administration of ACTH has been described as without effect on the blood levels, or to lead to transient increases in the urinary levels of the steroid (4, 5, 6, 7, 8).

Interpretation of the findings of different workers is difficult. The problem is somewhat complicated by the fact that ACTH appears to consist of several biologically active components (9, 10). The work of Bell and his collaborators is of special interest in this connection. These workers have partitioned a preparation of ACTH into several fractions with very high order of ACTH activity, as assayed in the adrenal ascorbic acid depletion test of Sayers *et al.* (10). However, the steroidogenic properties of the preparations have not been studied. This seemed to be of importance since evidence has recently been presented that the steroidogenic property of ACTH does not necessarily parallel the adrenal ascorbic acid depleting

Received October 8, 1957.

[1] This investigation was supported in part by a grant from the Cleveland Area Heart Society, and in part by a grant from the U. S. Public Health Service.

activity (9). Further, there is no evidence available to relate aldosterone stimulating activity to the effects on adrenal ascorbic acid. In order to clarify these points, Bell and his collaborators very kindly made available to us samples of two of their highly purified preparations (β and δ_1 corticotropins). These have been assayed for aldosterone and hydrocortisone stimulating properties in the decerebrate dog. Both preparations were found to stimulate hydrocortisone secretion to about the same extent. However, δ_1 corticotropin appears to be many times more active than β corticotropin in stimulating aldosterone secretion.

METHODS

In the assay of the first lots of β and δ_1 corticotropins, 1 mg. of the powdered preparations was weighed to the nearest 5 μg. and dissolved in 100 ml. of 0.9% NaCl made to 0.01N with HCl. The stock solution was frozen for storage. The frozen stock solution was thawed and an aliquot removed for assay on each assay day. In the assay of the second lots of β and δ_1 corticotropins, 50 to 100 μg. of the dried powders was weighed on each assay day, and dissolved in a suitable volume of 0.9% NaCl made to 0.01N with HCl. An aliquot of this freshly prepared solution was used for assay on the day of preparation.

To determine the effect of heating in alkali solution on the biological activity of δ_1 corticotropin, 15 ml. of a stock solution (20 μg./ml.) was adjusted to 0.1N with NaOH and heated in a boiling water bath for 20 minutes. The solution was divided into equal aliquots, corresponding to doses of 150 μg. of corticotropin. Each aliquot was adjusted to pH 7 and subsequently diluted to 1 liter of 0.9% NaCl made to 0.01 with HCl for assay.

For the assay, large male dogs were anesthetized with sodium pentobarbital and subjected to midcollicular decerebration. Decerebration was as follows: A flap of bone was raised on the dorsum of the skull. Bleeding from dural sinuses was controlled, and the dura was resected to expose the occipital and parietal lobes. The occipital lobes were removed with suction and the corpora quadrigemina were exposed and identified. The handle of a scalpel was passed through the brain stem at the midcollicular level. The brain substance rostral to the section was removed with suction. The pituitary was also removed. The internal carotid arteries and the posterior cerebral arteries were clamped. Spontaneous respiration was usually present; however, artificial respiration was routinely instituted by means of an alternating positive-pressure respirator to insure adequate oxygenation. Periodic rectal temperature observations were made and heat lamps or ice packs applied as necessary to maintain body temperature within the range 35–39°. Arterial blood pressure (femoral or carotid) was determined by means of a mercury manometer. A solution of norepinephrine or epinephrine in 5% glucose was infused when needed to maintain the blood pressure at or above 80 mm. Hg. Four hours after decerebration, the left adrenal vein was cannulated for collection of adrenal venous blood and the corticotropin to be assayed in one liter of 0.9% NaCl made to 0.01N with HCl, was infused over a period of approximately 2 hours. The adrenal venous blood collected during the infusion period was frozen for later steroid isolation. Aldosterone (as the diacetate) and hydrocortisone were isolated by paper chromatographic methods described in detail in earlier reports (11, 12).

One dog experiment served for the assay of only one dose of a given corticotropin, inasmuch as the entire adrenal venous blood sample from one experiment was required for accurate quantitation of the aldosterone secretion rate. The hydrocortisone secretion rate was determined on the same sample. In the first assay (Table 1) 18 dogs were used, and in the second (Table 2), 12 dogs.

(Assay I)

Table 1. Assay of steroidogenic activity of β and δ_1 corticotropins

A. *Stimulation of hydrocortisone secretion*

Dose, μg.		Response, μg. hydrocortisone/kg. body wt./hr.	
		Individual values*	Mean
β corticotropin	1	8.21, 7.48, 4.33	6.67
	5	8.15, 7.25, 24.80	13.4
	10	21.7, 20.4, 18.9	20.3
$\delta_1$corticotropin	1	1.09, 1.37, 6.17	2.87
	5	19.8, 10.6, 2.7	11.03
	10	23.5, 36.6, 18.0	26.03

s equals 7.01; b_c equals 17.03; λ equals 0.405; M equals −0.009; ±Sm equals 0.193; Potency δ_1/β equals 0.975 (0.51–1.53).

B. *Stimulation of aldosterone secretion*

Dose, μg.		Response, μg. aldosterone/100 kg. body wt./hr.	
		Individual values*	Mean
β corticotropin	5	1.92, 2.20, 5.60	3.24
	10	16.5, 9.7, 5.2	10.46
	100	17.3, 36.1, 11.5	21.63
δ_1 corticotropin	1	4.73, 7.36, 19.00	10.36
	5	12.1, 18.7, 9.25	13.35
	10	28.0, 51.4, 38.0	39.13

s equals 10.1; b_c equals 17.40; λ equals 0.576; M equals 1.210; ±Sm equals 0.299; Potency δ_1/β equals 16.23 (9.51–37.8).

b_c equals common slope of $\log_{10}$ dose response curve; s equals standard deviation of all the individual responses about this curve; λ equals s/b and is a measure of the accuracy of the assay; M equals logarithm of the ratio of potencies; Sm equals standard error of M.

* Each individual value represents the secretory rate of the steroid in one animal.

RESULTS AND DISCUSSION

The results of the two assays are presented in Tables 1 and 2. In assay I, a linear log dose relationship was found between doses of 1 to 10 μg. of both corticotropins, when the index of activity was hydrocortisone secretion (A, Table 1). The potency δ_1/β with respect to hydrocortisone secretion was 0.975 with limits[2] of 0.51 to 1.53. When aldosterone secretion was the index of activity, doses of 5, 10 and 100 μg. of β corticotropin were necessary to obtain a linear response, while doses of 1, 5 and 10 μg. of δ_1 corticotropin fell on the linear portion of the curve (B, Table 1). The potency ratio, δ_1/β with respect to aldosterone secretion, was 16.23 with limits of 9.51 to 37.8.

In assay II, in which a new lot of δ_1 corticotropin was assayed against the same sample of β, a different dose schedule was necessary. With respect to hydrocortisone secretion, doses in the range of $\frac{1}{4}$ to 16 μg. of both β and δ_1 corticotropin seemed to fall within the linear dose response relationship

[2] One standard error.

TABLE 2. ASSAY OF STEROIDOGENIC ACTIVITY OF β AND δ_1 CORTICOTROPINS (ASSAY II)

A. *Stimulation of hydrocortisone secretion*

Dose, μg.		Response, μg. hydrocortisone/kg. body wt./hr.	
		Individual values*	Mean
β corticotropin	1/4	4.90, 4.87	4.88
	1	14.0, 7.0	10.5
	4	21.8, 25.8	23.8
δ_1 corticotropin	1/4	4.97, 9.60	7.28
	4	25.0, 14.9	19.9
	16	28.3, 24.8	26.5

$s = 4.61$; $b_c = 11.7$; $\lambda = 0.395$; $M = 0.216$; $\pm Sm = 0.243$; Potency $\delta_1/\beta = 1.64$ (0.94–2.87).

B. *Stimulation of aldosterone secretion*

Dose, μg.		Response, μg. aldosterone/100 kg. body wt./hr.	
		Individual values*	Mean
β corticotropin	1/4	5.3, 10.4	7.85
	1	11.6, 17.7	14.65
	4	29.8, 32.4	31.1
δ_1 corticotropin	1/16	8.7, 16.9	12.8
	1/4	13.1, 15.4	19.25
	1	48.5, 24.3	36.4

$s = 7.95$; $b_c = 19.5$; $\lambda = 0.407$; $M = 0.8534$; $\pm Sm = 0.247$; Potency $\delta_1/\beta = 7.14$ (4.04–12.6).

b_c = common slope of $\log_{10}$ dose response curve; s = standard deviation of all individual responses about this curve; $\lambda = s/b$ and is a measure of the accuracy of the assay; M = logarithm of the ratio of potencies; Sm = standard error of M.

* Each individual value represents the secretory rate of the steroid in one animal.

(Table 2). When aldosterone secretion was used as the index of activity, doses of 1/16 to 1 μg. of δ_1 corticotropin and $\frac{1}{4}$ to 4 μg. of β corticotropin satisfied the criteria for linearity. The change in the dose schedule between assays I and II is unexplained, and appears to indicate a shift in the sensitivity of the assay. It is possible that variations in the physiological status (environmental temperature, degree of hydration, etc.) of the dogs may have influenced the sensitivity of the assay. The experimental design, however, permits a reliable estimate of relative potency since the steroidogenic activity of the one corticotropin was compared to that of the other in each assay, at the same time and under essentially the same conditions. The potency of the second lot of δ_1 as compared to β, with respect to hydrocortisone secretion, was 1.64 (limits 0.94–2.87). With respect to aldosterone secretion, the potency ratio δ_1/β was 7.14 (limits 4.04–12.6). Although the relative potency of the second lot of δ_1 corticotropin in stimulating aldosterone secretion was somewhat lower than that of the first lot, δ_1 corticotropin was again found to be more active than β in stimulating aldosterone output.

The variation in potency between the two lots of δ_1 corticotropin with

respect to aldosterone secretion raises the question of whether the aldosterone stimulating activity is due to a contaminant present to a varying extent in the two lots. δ_1 corticotropin is not a single substance, but contains several components (17). Preliminary electrophoretic studies of the δ_1 preparations in this laboratory have demonstrated four smaller components in addition to the major component. Assay has failed to reveal steroidogenic activity in any but the major component. However, the data thus far accumulated does not conclusively rule out the possibility that the aldosterone stimulating activity may be related to a minor component of δ_1 corticotropin.

It is known that δ_1 corticotropin is high in melanocyte stimulating activity (10). To determine if the aldosterone stimulating activity was related to the melanocyte stimulating activity, a sample of δ_1 corticotropin was heated (boiling water bath) in 0.1N NaOH for 20 minutes. This treatment is known to destroy corticotropic activity, but to leave intact or to potentiate melanocyte stimulating activity (10). The material was assayed at doses (150 μg.) greatly in excess of that necessary for maximal steroidogenesis. In the two experiments the hydrocortisone secretion rate was 5.7 μg./kg. body wt./hr. and that of aldosterone was 7.75 μg./100 kg. body wt./hr. These values indicate virtually complete loss of steroidogenic activity. The aldosterone stimulating activity is very probably not associated with the melanocyte stimulating activity.

The concept of separate regulating substances for the adrenal secretion of hydrocortisone and aldosterone finds considerable support in the finding of a dichotomy of biological activity of δ_1 and β corticotropins. There appears to be overlap of activity, since β corticotropin stimulates aldosterone secretion in the higher dose ranges. The nature of this overlapping of activity is not at all clear, although it may be related to the role of corticosterone as a common precursor of hydrocortisone and aldosterone (13).

The presence of an active aldosterone stimulating substance in ACTH raises the question of the possible role of this substance in the sodium retention observed clinically after ACTH administration. δ_1 corticotropin makes up about 10% by weight of mixed ACTH (10). Assuming a potency for δ_1 of approximately 10 times that of β corticotropin, it is possible to calculate that a combination of the two corticotropins in the proportions 1:9, δ_1:β, would have about twice the aldosterone stimulating activity of β alone. The implications of this observation to the preparation of ACTH for clinical use is currently being explored (14). It seems very likely that a large part of the disagreement among various workers as to the effect of ACTH on aldosterone output may be due to the presence of varying amounts of δ_1 corticotropin or an active aldosterone stimulating factor present as a contaminant.

The presence of a highly active aldosterone stimulating factor in aden-

hypophyseal tissue, which is the source of δ_1 and β corticotropins, remains an enigma. A large body of experimental evidence direct and indirect, in man, dog and the rat is available which indicates that the adenohypophysis is not the primary regulator of aldosterone output (15). Further, evidence has recently been presented that the diencephalon is the regulator of aldosterone secretion (12), and aldosterone stimulating activity has been found in saline extracts of beef diencephalon (16). Whether the diencephalic factor is the same as that in adenohypophyseal tissue cannot be answered at this time. It is possible that an aldosterone stimulating factor may be elaborated in the brain stem and stored in the pituitary gland in a manner analogous to that for the secretion of antidiuretic hormone. Further studies are clearly necessary before a definitive statement is possible.

Acknowledgments

We wish to express our thanks to Dr. Paul H. Bell of the Lederle Laboratories, Pearl River, N.Y., for the samples of purified corticotropins used in this study. We also wish to thank Robert C. Banks for generous and capable technical assistance.

Addendum

Dr. Paul Bell advises us that the two samples of δ_1 corticotropin which he supplied for these assays were taken from the same original lot. Differences in potency estimates thus remain unexplained. Possibilities include 1) decomposition in the solid state and 2) inherent variability in the dog assay. The latter seems the more likely. Dr. Bell also points out that alkali treatment drastically changes the properties of the intermedin originally present in the δ_1 fraction (potentiates and extends the duration of action). For this reason, these experiments do not necessarily rule out the possibility that the aldosterone stimulating activity is associated with the short acting intermedin present in δ_1 corticotropin.

REFERENCES

1. Farrell, G. L., E. W. Rauschkolb and P. C. Royce: *Am. J. Physiol.* **182:** 269. 1955.
2. Singer, B. and M. P. Stack-Dunne: *J. Endocrinol.* **14:** 1086. 1954.
3. Giroud, C. J. P., A. V. Schally, J. Stachenko and E. H. Venning: *Proc. Soc. Exper. Biol. and Med.* **92:** 855. 1956.
4. Liddle, G. W., J. Cornfield, A. G. T. Casper and F. C. Bartter: *J. Clin. Endocrinol. and Metab.* **15:** 852. 1955.
5. Simpson, S. A. and J. F. Tait: *Recent Progress in Hormone Res.* **11:** 183. 1955.
6. Axelrad, B. J., B. B. Johnson and J. A. Luetscher, Jr.: *J. Clin. Endocrinol. and Metab.* **14:** 783. 1954.
7. Venning, E. H., A. Cartallera and I. Dyrenfurth: *J. Clin. Endocrinol. and Metab.* **14:** 784. 1954.
8. Muller, A. F., A. M. Riondel and E. L. Manning: *Lancet* Nov. **17:** 1021. 1956.
9. Stack-Dunne, M. P. and F. G. Young: *Ann. Rev. Biochem.* **23:** 405. 1954.

10. Bell, P. H.: *J. Am. Chem. Soc.* **76:** 5565. 1954.
11. Farrell, G. L., P. C. Royce, E. W. Rauschkolb and H. Hirschmann: *Proc. Soc. Exper. Biol. and Med.* **87:** 141. 1954.
12. Rauschkolb, E. W. and G. L. Farrell: *Endocrinology* **59:** 526. 1956.
13. Tait, J. F.: *Personal communication.*
14. Stack-Dunne, M. P.: Personal communication.
15. Gaunt, R., A. A. Renzi and J. J. Chart: *J. Clin. Endocrinol. and Metab.* **15:** 621. 1955.
16. Farrell, G. L., E. W. Rauschkolb, R. B. Fleming and F. Yatsu: Proc. 39th Meet. Endocrine Society, New York, N. Y., 1957.
17. Farrell, G. L.: Unpublished observations.

IN VITRO THYROXINE EFFECT ON RAT KIDNEY CORTEX AS INFLUENCED BY ADDITION OF AMINO ACIDS[1]

R. H. LINDSAY AND S. B. BARKER

Department of Pharmacology, University of Alabama Medical Center, Birmingham, Alabama

ABSTRACT

The thyroxine maintenance of the metabolism of rat kidney slices incubated at 5° C. for 24, 48 and 72 hours has been studied in terms of specific participation of some 50 amino acids and related compounds.

Based on a comparison of initial Q_{O_2} and maintenance with similar values obtained with simple Ringer-glucose solution without added amino acid, DL-alanine, N-acetyl-DL-alanine, and L-proline were the most active. Considered as intermediate were β-alanine, threonine, β- and γ-amino-n-butyric acids, taurine, α- and β-amino-isobutyric acids. Tryptophan inhibited the thyroxine effect, alone or in the presence of alanine or proline.

Further analysis of the DL-alanine action indicated the D-form to be responsible for all of the activity. Indirect evidence points to deamination plus decarboxylation being important in the alanine effect, but transamination and even peptide bond synthesis have not been ruled out.

WHEN kidney slices prepared from thyroidectomized rats were held at 5° C. in Krebs-Ringer-phosphate-glucose solution, the presence of 5–10 μg. thyroxine per ml. allowed much better metabolic maintenance of the tissue than was seen in the absence of the hormone (1). Substitution of glycine-NaOH for the phosphate buffer did not alter the control tissues, but thyroxine then gave nearly 100% maintenance at 24 hours. When Ringer-DL-alanine-glucose was employed, control respiration was considerably improved and the tissue with thyroxine was somewhat better than 100% at 24 hours, 100% or better at 48 hours, and well maintained at 72.

Although the buffering capacity of peptides of glycine and alanine is considerably greater than that of the simple amino acids, such compounds as glycylglycine, glycylglycylglycine and alanylalanine were no more effective than phosphate in supporting the thyroxine effect. Because of this seemingly specific action of the two free amino acids, trials have been made of most obtainable amino acids and related nitrogenous compounds.

Received October 18, 1957.

[1] Supported by research grants from the American Cancer Society and the National Institutes of Health, United States Public Health Service (A-1545).

EXPERIMENTAL METHODS

Male and female albino rats of the Sprague-Dawley strain were used not less than six weeks after thyroidectomy. The animals were sacrificed by exsanguination from the abdominal aorta under light ether anesthesia. Both kidneys were quickly removed and placed in cold Krebs' Ringer solution. Thin slices of cortex were prepared using the Deutsch technic.

The basal medium for both measurement of metabolism and incubation was Krebs' Ringer solution containing 200 mg.% glucose. The magnesium content was doubled, since studies showed that this change gave somewhat better results at 48 and 72 hours without altering the starting Q_{O_2}. To 9 volumes of this solution was added 1 volume of a 0.2*M* solution of an amino acid or related compound previously adjusted with NaOH to a pH of 7.5–0.05. For the more insoluble amino acids, it was necessary to dissolve the compound in enough Ringer-glucose solution to yield a concentration of 0.02 to 0.04*M*. After the pH had been adjusted to 7.5 with alkali, further Ringer-glucose was added as necessary to bring the amino acid concentration to 0.02*M*. Tyrosine and cystine were so insoluble that even 0.02*M* could not be achieved below a pH of 10.

A stock solution of L-thyroxine[2] containing 100 μg./ml. was made by dissolving the hormone in enough 0.1N NaOH to give a final concentration of 0.005N. Before diluting this to final volume, it was made isotonic to 0.9% NaCl by addition of the proper amount of 10% NaCl.

Each Warburg flask contained 2.7 ml. of Ringer-glucose-amino acid solution to which was added 0.3 ml. of either the thyroxine solution or a control solution of 0.005N NaOH in 0.9% NaCl (indicated as "T" or "B1," respectively, in the table and figures). The final thyroxine concentration was thus 10 μg./ml.

Solutions were freshly prepared on the day of the experiment. The flasks were dried at 110° C; otherwise, only moderately aseptic technics were used. Several bacterial counts made at the end of the three-day incubation period were found to be in the range of 500 to 5,000 per ml., and were the same in control and thyroxine vessels.[3]

The procedure followed for all experiments consisted of a preliminary determination of oxygen consumption at 37° for one hour, following an equilibration period of 15 minutes. The vessels were then detached from the manometers, flushed with oxygen, capped with squares of Parafilm and stored at 5° without shaking. Twenty-three hours later, the vessels were regassed with oxygen, remounted on the manometers, equilibrated for 15 minutes and oxygen consumption again determined over one hour at 37°. They were then returned to 5° storage, and the procedure repeated at 48 and 72 hours. All Q_{O_2} values are $\mu l.O_2$/mg. wet weight of tissue/hr.

RESULTS AND DISCUSSION

Figure 1 shows the results of a series of experiments to determine the contribution of the glucose and of the DL-alanine to the thyroxine effect. In the case of Ringer alone (no added substrate), the initial Q_{O_2} was only 2.29, but thyroxine maintained respiration at a level higher than the blank on all three days of incubation. When glucose was added to the Ringer solution, there was a slight increase in initial Q_{O_2} and somewhat better maintenance for all three days in the thyroxine vessels.

[2] Generously provided by Dr. H. L. Fevold, of the Travenol division of Baxter Laboratories.

[3] Thanks are due Dr. C. H. Winkler of the Department of Microbiology, for these determinations.

The addition of alanine to the Ringer solution istead of glucose produced a marked increase in starting oxygen consumption, which was well maintained for the first day even in the absence of thyroxine. After two and three days of incubation, respiration in the control vessels was very low, as though some essential material had been exhausted or some toxic substance produced. Oxygen consumption in the thyroxine vessels was excellent for two days, and then in turn plummeted on the third. The addition

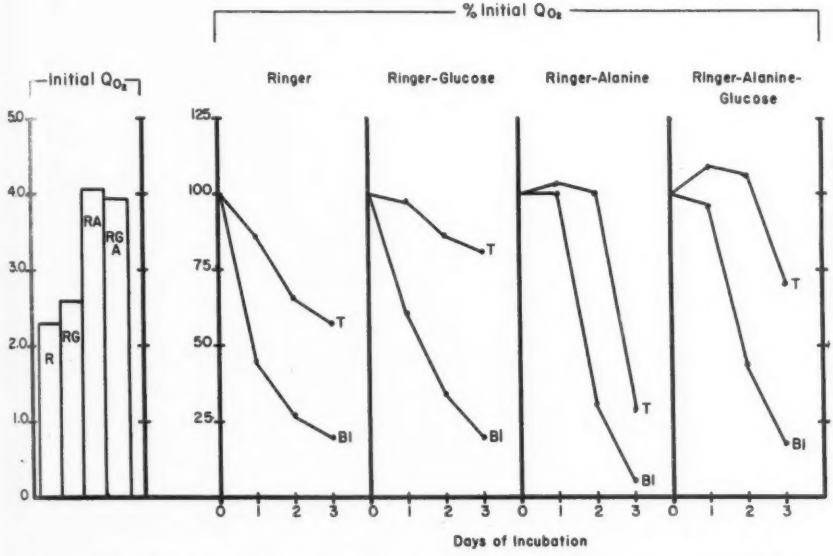


FIG. 1. Effects of DL-alanine and glucose on the oxygen consumption of kidney slices incubated in Krebs' Ringer solution. Oxygen uptake was measured at 37° C initially and after 1, 2 and 3 days of incubation at 5° C. Thyroxine concentration ("T") was 10 μg./ml.

of glucose to the Ringer-DL-alanine system did not alter the starting Q_{O_2} and showed only minor effects on the incubated blanks. However, Ringer-alanine-glucose in the presence of thyroxine gave somewhat better than starting metabolism after one and two days of incubation, and far better third day effects than with alanine alone.

These findings with the Ringer, Ringer-glucose, Ringer-alanine and Ringer-glucose-alanine series have been used in setting up criteria for examining the results of the extensive trials with other amino acids. These data are shown in Table 1, with the nitrogenous compounds arbitrarily grouped as "Most Active," "Intermediate" and "Inactive." All substances producing an initial rate of oxygen uptake above 2.5, about that of Ringer-glucose, and maintaining it in the presence of thyroxine as well as the Ringer-glucose were considered as belonging in the first two active cate-

TABLE 1. OXYGEN CONSUMPTION OF KIDNEY SLICES IN THE PRESENCE OF VARIOUS AMINO ACIDS AND RELATED COMPOUNDS

	Initial Q_{O_2}*		% of initial Q_{O_2} after					
			24 hours		48 hours		72 hours	
	B1	T	B1	T	B1	T	B1	T
Most Active								
DL-Alanine (35)†	3.94	3.92	96	109	43	106	18	70
L-Proline (7)	3.93	3.81	64	112	17	105	11	97
N-Acetyl-DL-Alanine (2)	3.72	3.77	70	115	25	103	12	72
Intermediate								
β-Alanine	3.24	3.95	54	107	24	90	10	75
DL-Threonine	3.12	2.80	54	109	18	98		
γ-Amino-n-butyric (2)	3.10	2.88	65	102	32	92	22	82
β-Amino-n-butyric (2)	2.87	2.55	77	104	38	95	23	88
Taurine	2.76	2.90	82	99	39	90		
β-Amino-isobutyric	2.60	2.50	68	103	32	97	8	87
α-Amino-isobutyric	2.46	2.35	81	108	35	100	16	92
Inactive								
DL-Norleucine	4.97	4.84	49	80	15	51	5	38
DL-Aspartic acid	4.55	3.87	64	97	23	76	9	41
DL-Norvaline	4.29	4.19	60	101	18	74	5	47
DL-Isoleucine	4.21	3.90	54	94	18	75	7	63
Cysteine	4.14	4.17	15	26	3	19	2	16
DL-Leucine	3.94	3.73	75	98	24	80	6	67
N-Acetyl-glycine	3.72	3.78	80	100	30	83	12	28
DL-Valine	3.66	3.37	60	101	18	77	5	58
DL-Asparagine (2)	3.51	3.48	67	112	34	86	19	55
Glutamine	3.55	3.27	59	118	24	83	9	26
Methionine	3.48	3.23	47	64	14	42	7	29
DL-Ornithine HCL (2)	3.46	3.68	51	98	26	81	14	68
DL-Lysine HCL	3.32	2.98	38	88	9	65	4	45
Glutamic acid	3.29	3.49	42	112	15	59	6	26
Hydroxy-L-Proline	3.25	3.31	40	81	12	57	8	41
Carnosine	3.20	3.54	37	81	14	74	10	53
D-Histidine HCL	3.12	3.09	18	75	4	55	3	31
Serine	3.09	3.38	43	89	21	77	18	39
DL-Phenylalanine	3.06	2.83	77	83	36	60	11	42
Glycine (10)	3.02	3.00	50	97	17	79	8	70
L-Histidine	3.02	2.94	21	81	4	65	3	49
α-Amino-n-butyric acid (2)	3.00	2.80	67	75	30	62	17	53
DL-Sarcosine	2.90	2.75	64	102	28	87	20	75
Citrulline (2)	2.77	2.61	67	99	31	83	15	74
Ethionine	2.76	2.53	49	56	15	26	7	28
Betaine (2)	2.65	2.44	73	100	36	92	21	81
Acetamide	2.64	2.58	63	103	35	84	20	78
Ethylenediamine	2.63	2.90	29	32	10	19	8	15
Purine	2.60	2.67	45	73	21	49	14	38
Urethane	2.58	2.45	66	102	31	88	15	77
Urea (4)	2.57	2.55	67	100	35	87	21	82
Thiourea	2.57	2.51	54	77	31	48	26	44
L-Arginine HCl	2.52	2.46	31	83	21	74	18	69
L-Phenylalanine	2.39	2.28	58	94	23	75	5	61
L-Tryptophan (3)	1.99	1.90	44	40	40	35	20	35

* See text for complete explanation.
† Number of experiments shown in parentheses when more than one.

gories. Those failing in either aspect of starting metabolic rate or maintenance were labeled inactive. Of the active compounds, three were selected as being outstanding on the basis of both starting Q_{O_2} and thyroxine effect. These were DL-alanine, L-proline and N-acetyl-DL-alanine. Special comments about each of these can be made.

Most Active Amino Acids. More experiments have been run with DL-alanine than any other amino acid. The DL-form was employed as being the most readily available. Effects in the presence of thyroxine have occasionally been as high as 130% of the starting value at 24 hours, with 100% and 95% at 48 and 72 hours, respectively. As the data in Table 1

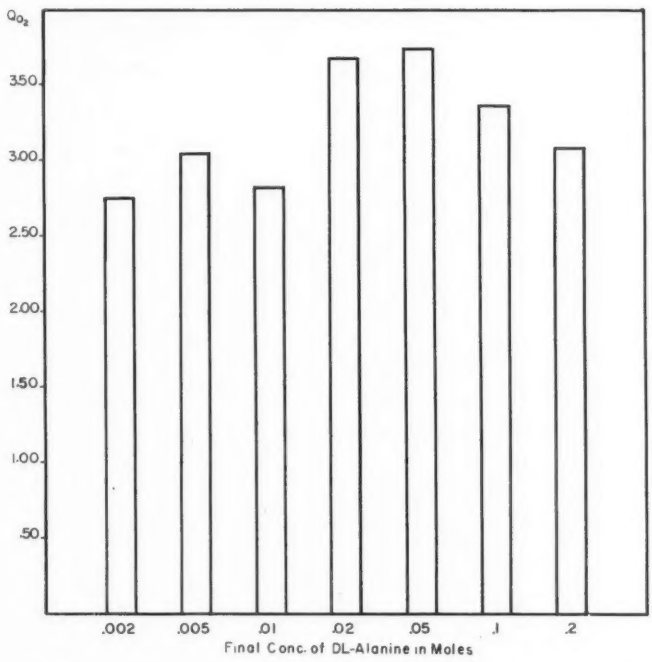


FIG. 2. Effects of increasing concentrations of DL-alanine added to Krebs' Ringer-glucose on initial oxygen consumption of kidney slices.

indicate, the average results have not been this good. Variabilities in thyroxine preparations are believed to be responsible for some of the differences, since use of a more highly purified thyroxine, judged chromatographically, recently has shown a return towards the earlier findings, especially with regard to 72 hour maintenance. Studies are continuing in an attempt to delineate more clearly the causes for the decrease at 72 hours.

Figure 2 shows that increasing the concentration of DL-alanine in the medium increased the starting oxygen consumption to a plateau at 0.02 to 0.05*M*, beyond which levels the Q_{O_2} fell off. When the effect of varying alanine concentrations was studied on long-term incubation (Fig. 3) 0.01 to 0.05*M* were found to be essentially plateaued, with 0.02*M* slightly

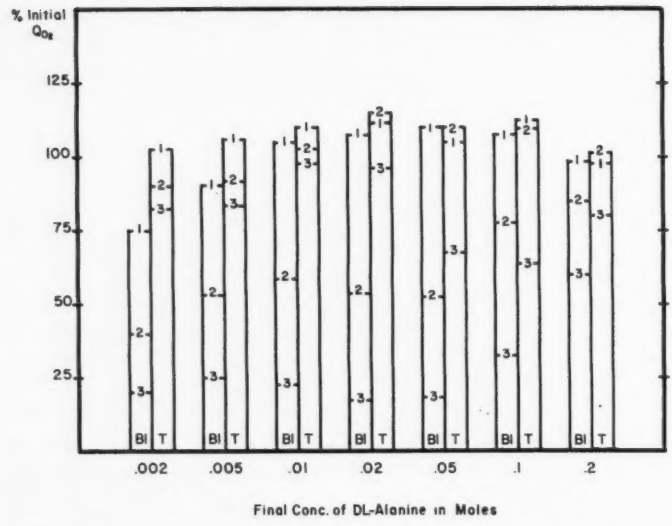


FIG. 3. Effects of increasing concentrations of DL-alanine on oxygen consumption of kidney slices incubated in Krebs' Ringer-alanine-glucose for 1, 2, and 3 days, as indicated by the numerals shown at the top of each appropriate bar. Thyroxine concentration ("T") was 10 μg./ml. See text for complete description of conditions of incubation.

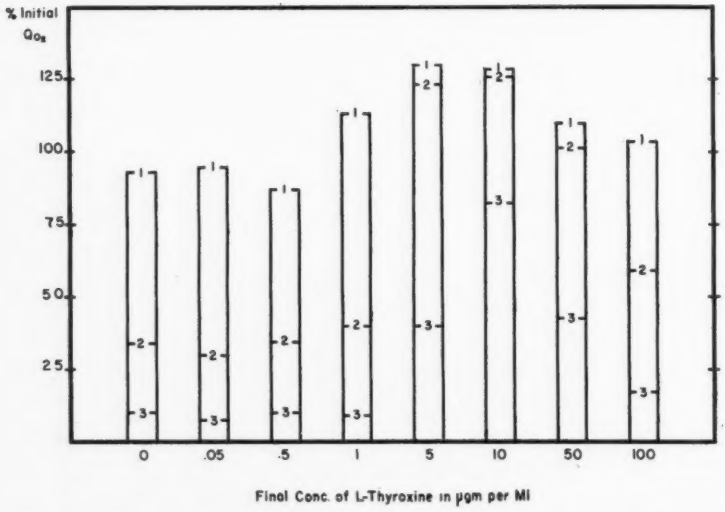


FIG. 4. Effects of increasing concentrations of L-thyroxine on oxygen consumption of kidney slices. Incubation was carried out in Krebs' Ringer solution with DL-alanine 0.02*M*, glucose 200 mg.%, for 1, 2 and 3 days, as indicated by the numerals shown at the top of the appropriate bars.

superior to either of the others. This was the actual basis for the selection of 0.02*M* as the testing level for all amino acids. Optimal thyroxine concentrations were also determined with alanine present, as shown in Figure 4. No effect was seen until 1 μg./ml. was reached. An increase to 5 and 10 μg./ml resulted in improved effects, especially at 48 and 72 hours. Con-

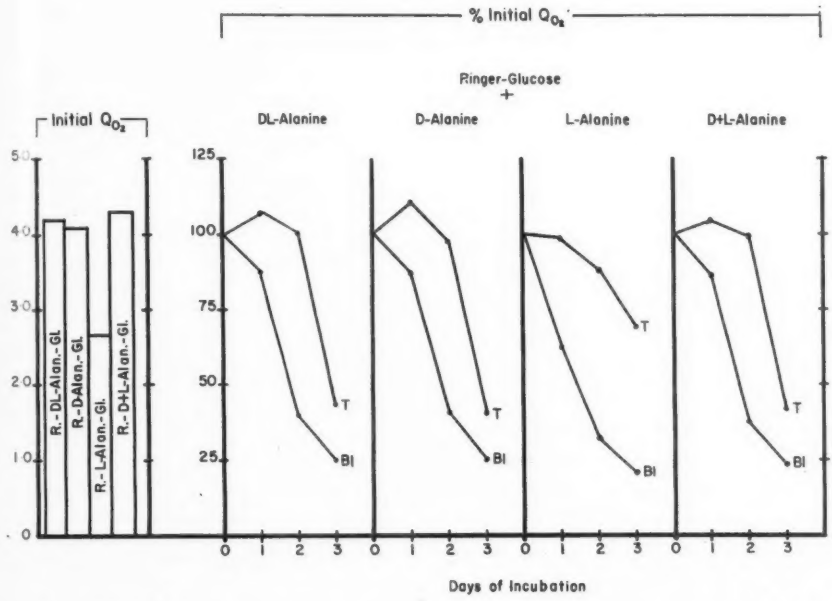


FIG. 5. Effects of the optical isomers of alanine on oxygen consumption of kidney slices incubated in Krebs' Ringer-alanine glucose solution. Each isomer was present at a final concentration of 0.01*M*. The racemic mixture indicated as "D+L-alanine" was made by mixing equal volumes of 0.02*M* solutions of each isomer. Oxygen uptake was measured at 37° C initially and after 1, 2 and 3 days of incubation at 5° C. Thyroxine concentration ("T") was 10 μg./ml.

centrations beyond this level, 50 and 100 μg./ml., gave less marked results but still were better than the blanks.

In further analyzing the role of DL-alanine, the enantiomorphs were tried separately, as shown in Figure 5. As would be expected from the high activity of D-amino acid oxidase present in kidney tissue (2), the initial Q_{O_2} with D-alanine was 55% higher than that with the L-form. When the racemic mixture, either as obtained or as made from the optically active forms, was used at 0.02*M*, the starting Q_{O_2} was the same as with 0.01*M* L-alanine. Maintenance of metabolism during prolonged incubation with thyroxine was also practically the same for these three solutions at the concentrations mentioned. Since 0.01*M* L-alanine was equivalent to the

Ringer glucose at all times, it may be concluded that the activity of DL-alanine is entirely due to the D-isomer and furthermore that the L-isomer does not interfere. This last point is important, since most of our experiments with alanine have employed the more readily available DL-form.

Since N-acetyl-DL-alanine was practically as good as the non-acetylated material, deacetylation may have taken place prior to any further action, although it is conceivable that direct deamination of the N-acetylated amino group occurred. Chromatography of the incubation medium failed to reveal any free alanine present after the three-day incubation, but that absence may simply indicate that deacetylation is the rate-limiting activity. Any free alanine formed would then be altered instantly.

L-Proline has given more consistent results after 72 hours of incubation than DL-alanine and has aroused our interest for several reasons. It is a cyclic imino acid instead of an amino, the L-form is very active, and its metabolism is known to yield glutamic acid (3,4), which has proven inactive in this study. Production of hydroxyproline probably is not involved since it, too, is inactive. Although chromatography of our incubated kidney slices has shown the presence of glutamic acid, no hydroxyproline has been found. Further inquiry into the specific role of proline is limited by the lack of availability of the D-form, but DL-proline did not support a thyroxine effect as well as did L-proline.

Intermediate Amino Acids. Like the most active compounds, these also comprise a mixed group, including three β- and one γ-amino acids, an hydroxy-amino acid and an aminoethylsulfuric acid. Again, it is difficult to find any reaction product or metabolic process which could be common to such an heterogeneous group. This may be further support for the concept that several reactions are involved in the thyroxine "activation."

Inactive Amino Acids. Among the compounds placed in this category are several supporting oxygen consumption at a far greater rate than the Ringer-glucose alone, but which fell off rapidly. It is realized that our classification may be so arbitrary as to rule out many interesting contributions, especially the ones with intial Q_{O_2}'s of 4.0 and above. Since the primary concern has been over the thyroxine effect, such a selection is believed justifiable. Nonetheless, brief attention may be directed towards norleucine, aspartic acid, norvaline and isoleucine.

Special mention should be made of tryptophan, since it was unique in not permitting any thyroxine effect until the third day and even then only a small difference between blank and thyroxine appeared. In pursuit of this phenomenon, it has been found that D- or L-tryptophan blocks thyroxine action in the presence of either alanine or proline. In fact, when both L-tryptophan and alanine were present in equimolar amounts, thyroxine caused a slight depression in oxygen consumption below the control values. Further study of this is being made.

GENERAL DISCUSSION

Amino acids are known to undergo a variety of reactions, including peptide bond synthesis, deamination and transamination. Our analysis of the amino acid contribution to thyroxine maintenance of kidney metabolism has not yet progressed to the point that we can even decide the energetics involved, but a few comments can be made. Preliminary experiments indicate that there is no incorporation of C^{14}-tagged alanine in the protein of the incubated tissue. Extensive chromatographic study has revealed no consistent formation of an iodine-containing complex such as a peptide of thyroxine or one of its derivatives. The possibility is, therefore, remote that a synthetic reaction is the key.

On the other hand, both ammonia production and a pH rise have been encountered during incubation. It is strongly suspected that the combined toxicities of increasing pH and the ammonium ion itself are important factors in decreasing the metabolic activity of the tissue. Studies are continuing to determine the importance of decarboxylation of pyruvate resulting from alanine deamination and metabolism of the resulting two-carbon residue. Even less exploration has been carried out with the proline.

The well-established presence of highly active D-amino acid deaminases in kidney tissue undoubtedly explains the great activity in our system of D-alanine. We have not yet evaluated D-proline, but it is known that the L-form of this heterocyclic imino acid can be opened to yield glutamic acid. If this occurs, there must be a special reactivity of the "glutamate" formed, in contrast to that added to the incubation medium as preformed glutamate.

It should be pointed out that many of the amino acids on the "Intermediate" and "Inactive" lists undergo active deamination, transamination and peptide bond reactions. There is no indication at present as to the specific reactions distinguishing alanine and proline from the rest. That small amount of activity which some of these do show may result from the production of alanine or of some common metabolic product, but at a lower concentration than the added $0.02M$. It may be noted that neither of the two is an "essential" amino acid, a fact which implies a considerable degree of interconvertibility with other amino acids.

REFERENCES

1. BARKER, S. B.: *Ciba Foundation Colloquia on Endocrinology* **10**: 253. 1957.
2. KREBS, H. A.: *Biochem. J.* **29**: 1620. 1935.
3. WEIL-MALHERBE, H. AND H. A. KREBS: *Biochem. J.* **29**: 2077. 1935.
4. TAGGERT, J. V. AND R. B. KRAKAUR: *J. Biol. Chem.* **177**: 641. 1949.

COMPARATIVE STUDY OF LUTEOTROPIN SECRETION BY HYPOPHYSIAL AUTOTRANSPLANTS IN THE RAT. EFFECTS OF SITE AND STAGES OF THE ESTRUS CYCLE[1,2]

MIROSLAVA NIKITOVITCH-WINER AND JOHN W. EVERETT

Department of Anatomy, Duke University School of Medicine, Durham, North Carolina

ABSTRACT

The persistence of luteotropin (LTH) secretion in pituitary grafts was studied in 47 rats. In 14 the pars distalis was autografted to the anterior chamber of the eye on the day of estrus. In the 9 animals in which the graft "took," secretion of LTH was demonstrated.

When the pars distalis was autografted to the kidney on various days of the cycle (2 animals on the day of estrus, 7 on the second day of diestrus, 9 on the third day of diestrus and 15 in proestrus), secretion of LTH was found in all cases. This was not at first recognizable in the last group, for luteolysis occurs during proestrus and the corpora lutea had lost their capacity to respond. Once new corpora lutea were produced by gonadotropin injection, LTH secretion was demonstrable.

In all cases LTH secretion was indirectly measured through the ability of uteri to respond to trauma with a decidual reaction. Despite the fact that no estrogen secretion was evident in the engrafted rats, uteri retained a reactivity to trauma for as long as 42 days.

It is concluded that luteotropin is secreted by pituitary grafts regardless of the time of the cycle when the pituitary is transplanted or of the site in which it is placed.

ADENOHYPOPHYSIAL grafts in sites remote from the hypothalamus have repeatedly failed to maintain gonadal function. Thus, complete gonadal atrophy has consistently been reported in rats bearing pars distalis grafts in the anterior chamber of the eye (1, 2, 3, 4, 5, 6). At the present time it is generally believed that FSH and LH secretions are indeed interrupted when the pituitary gland is placed away from the hypophysial portal vessels. On the other hand, it has been demonstrated that the gland is able to secrete luteotropin (LTH) under such conditions. Desclin (7) believed that this was possible only if a large quantity of estrogen (Stilbestrol) was administered to the experimental animal. Estrogen, he

Received October 18, 1957.

[1] This investigation was supported in part by grants from the Committee for Research in Problems of Sex, National Academy of Sciences-National Research Council and from the Research Council of Duke University.

[2] This publication represents a part of the dissertation submitted by M.N-W. in partial fulfiliment of the requirements for the degree of Doctor of Philosophy.

thought, directly stimulates the graft to secrete luteotropin. However, Everett later demonstrated conclusively in both short-term and long-term experiments (8, 9) that LTH is secreted by adenohypophysial transplants placed in the kidney without any attendant special stimulation. Recently Desclin (10) confirmed this. Both he and Everett confined their transplantation operations to the day after ovulation as standard procedure.

The present experiments were conducted to answer two primary questions: whether pituitary grafts in the anterior chamber of the eye are as capable of LTH secretion as grafts in the kidney; and, whether the time of the cycle at which the hypophysis is transplanted influences the subsequent secretory capacity of the gland. As the experiments proceeded several secondary questions arose. For how long after pituitary transplantation can the uterus respond with the decidual reaction to trauma? This reactivity had been observed (8) during only the 8-day periods of the short-term transplantation series. Can such tests be repeated from time to time? In normal pseudopregnant rats (11, 12) the decidual reaction fails when the uterus is traumatized after day 7. In the circumstance of no decidual reaction and the corresponding lack of corpus luteum function, would luteotropin secretion nevertheless be demonstrable after the artificial induction of new corpora lutea?

MATERIALS AND METHODS

The experimental animals were 47 adult female rats from our inbred Vanderbilt (Osborne-Mendel) strain, 4 to 7 months old at the beginning of the experiments. The animals at that time weighed between 180 and 250 gm. They were kept in an air-conditioned room that usually ranged from 75°–80° F. The lights were controlled by a time switch so that they were "on" for 14 hours and "off" for the next 10 hours. When the hour of the operation is stated, it is always given in "colony time," reckoned from "midnight" as 5 hours after the lights are turned off. The basic diet consisted of Purina dog chow and water *ad libitum*. After they were operated on, the animals received a supplement of milk 3 times a week and brewers' yeast once a week. Five per cent dextrose was added to the drinking water.

Vaginal smear history was recorded at least 2 weeks pre-operatively and throughout the post-operative period, six days each week. The prediction of the length and various stages of the cycles in progress at the time of operation was based on the preoperative history. Following the transplantations, the smears remained of the diestrus type, except for transient estrogenic responses to the FSH-LH injections in Series II-B-1 (see below).

In the group of 14 animals in which the anterior chamber of the eye was the chosen site for the pituitary graft (Series I) the auto-transplantation of the gland was always performed on the day of estrus. The animals in which the gland was transplanted to the kidney at different stages of the cycle (Series II) were distributed into 3 different groups. Thus, Series II-A consists of 2 animals operated on during the day of estrus and 7 animals during the second day of diestrus. Series II-B-1 comprises 15 animals operated on during the day of proestrus of a 4-day cycle. Series II-B-2 consists of 9 animals in which the transplantation was performed on the third day of diestrus in a cycle of 5-day duration. A prerequisite for any animal to be used in either part of Series II-B was that at least 2 vaginal cycles in sequence preceding the operation had to be of uniform length.

The techniques of hypophysectomy and transplantation of the hypophysis into the kidney were exactly as described by Everett (8, 9). When the pituitary was to be transplanted to the anterior chamber of the eye, once hypophysectomy had been accomplished, a small slit was made with a fine knife near the sclero-corneal junction and the pituitary was introduced through the slit. Because of the restricted operating area, the gland was manipulated considerably more during transplantation to the eye than during transplantation under the kidney capsule.

Luteotropin secretion was tested indirectly by its effect on progesterone secretion by the corpus luteum, as made evident by the deciduoma reaction to uterine trauma. In most animals, this test was performed on 2 occasions. First the right uterus was traumatized at 5 to 14 days following the transplantation, and the left one at 17 to 70 days. The standard procedure was to tie 2 loops of silk thread through the antimesometrial border of the uterus. Four days later, the degree of decidual reaction was evaluated on a scale of 4 by the amount of swelling that had occurred around the loops. In cases of minimal or moderate reaction histological proof was demanded.

In Series II-B-1, after no decidual reaction was obtained in the first test, new corpora lutea were artificially induced by injections of FSH and LH. The FSH injections were begun 23 to 67 days after pituitary transplantation. Depending on the hormone preparation used, 1 to 5 mg. was injected subcutaneously twice daily during a period of 3 days. This was followed the fourth day by an injection of LH.[3]

At the end of each experiment each animal was killed with an overdose of Nembutal. The reproductive tract and the pituitary grafts were examined *in situ* before removal. All pituitary grafts to the eye, numerous pituitary grafts to the kidney, most ovaries and some uteri with questionable decidual reactions were fixed in Bouin's fixative.

The pituitary capsules, in all cases, were searched very carefully with a dissecting microscope (×7). In the majority of animals this region was studied histologically in serial sections. For this purpose, the cranial floor was fixed in either Bouin's solution for 5 to 6 weeks or Heidenhain's SUSA overnight. Decalcification was accomplished in the former instance by the prolonged action of the fixing agent and in the latter by immersion in 5% trichloroacetic acid for 48 to 72 hours.

All tissues intended for histological examination were imbedded in paraffin, serially sectioned, and stained with a modified Mallory trichrome technique.

When the sections of pituitary capsules were examined it was found that in most cases no pars distalis remnants were present. When any glandular residue remained it was always very small, composed of clusters of only a few cells which appeared undifferentiated. In the nature of the present experiments such residues would be of no consequence to the end result.

RESULTS

DECIDUAL RESPONSE TO UTERINE TRAUMA. SERIES I. Pituitary autografts to the anterior chamber of the eye. A. *Animals with functional grafts.* The deciduoma test was performed on two occasions in all 9 animals of this series, 5 to 13 days and 31 to 70 days, respectively, after transplantation. A positive response to the first trauma would demonstrate the presence of LTH secretion, and the response to the second trauma

[3] For various FSH and LH preparations we are indebted to Dr. W. H. McShan of the Department of Zoology, University of Wisconsin, to Dr. J. A. Green, Department of Anatomy, University of North Carolina, and to Dr. Irby Bunding of the Armour Laboratories.

would show that the transplant is capable of a prolonged secretion of that hormone. The first trauma was followed in all animals by a maximal decidual reaction (Table 1-A). After the second trauma, however, the results were less uniform. Two animals responded maximally, 3 moderately and 4 not at all. This indicates that although eye grafts are capable of originally secreting adequate amounts of LTH, some appear to deteriorate with time and to fail to secrete enough of this hormone to continue corpus

TABLE 1. PITUITARY AUTOGRAFTS TO THE ANTERIOR CHAMBER OF THE EYE (SERIES I)

A. *Animals with functional grafts*					B. *Animals with non-functional grafts*		
Animal No.	Days, operation to 1st trauma	Decidual response	Days, operation to 2nd trauma	Decidual response	Animal No.	Days, operation to trauma	Decidual response
1	12	++++	40	++++	1	5	0
2	10	++++	31	++++	2	12	0
3	5	++++	42	++ & +++	3	12	0
4	6	++++	36	++	4	13	0
5	10	++++	31	++	5	10	0
6	13	++++	41	0			
7	5	++++	42	0			
8	10	++++	36	0			
9	9	++++	70	0			

luteum function. B. *Animals with non-functional grafts.* In each of 5 animals one traumatization was performed 5 to 13 days after transplatation and none of the uteri responded with a decidual reaction (Table 1-B).

SERIES II. Pituitary autografts to the kidney capsule on various days of the estrus cycle. A. *Transplantation on the day of estrus or second day of diestrus.* Two of the 9 animals in this group were subjected to transplantation on the day of estrus (stage III) and the other 7 on the second day of diestrus (stage V_2). Maximal decidual responses were elicited in either case. Therefore, all 9 were grouped together and there was no need to increase the number of animals in the estrus group itself. In the majority of animals the uterine trauma was performed on two occasions, 3 to 14 days and 17 to 37 days, respectively, after the pituitary was transplanted. The decidual reponse was pronounced and approximately equivalent on both occasions, as shown in Table 2. Thus these kidney grafts adequately secreted LTH throughout the length of the experiment. B. *Transplantation during proestrus or late diestrus.* This group can be divided into two subgroups, in both of which the actual time in days between the last ovulation and transplantation was identical, but which differed as follows: Series II-B-1 consists of animals with a history of 4-day cycles. Their anterior pituitaries were transplanted on the day of proestrus. Series II-B-2 was restricted to animals with cycles of 5-day duration. Here the pituitary was transplanted on the third day of diestrus. The respective results are the following: Series

TABLE 2. PITUITARY AUTOGRAFTS TO THE KIDNEY CAPSULE. TRANSPLANTATION ON THE DAY OF ESTRUS OR SECOND DAY OF DIESTRUS (SERIES II-A)

Animal No.	Stage of cycle	Days, operation to 1st trauma	Decidual response	Days, operation to 2nd trauma	Decidual response
1	III	8	++++	22	++++
2	III	13	++++	37	++++
3	V_2	13	++++	37	++
4	V_2	3	++++	17	++++
5	V_2	3	++++	17	++++
6	V_2	11	+++	35	+++
7	V_2	11	++++	35	+++
8	V_2	6	+++	—	—
9	V_2	7	++++	—	—

II-B-1. In the 15 animals in this group, the operations for removal of the adenohypophysis and its subsequent grafting to the kidney capsule were begun between 8:30 A.M. and 1:45 P.M. All were completed before the expected occurrence of LH release (13). Four to 21 days following this operation the right uteri of all animals were traumatized. Deciduomata failed to occur in 12 of the 15 animals. To test the possibility that the grafts were nevertheless secreting LTH, new corpora lutea were artificially induced by injections of FSH and LH. There were 8 acceptable experiments in which the injections induced new corpora lutea. In every one of these there was a moderate-to-maximal decidual response to the second trauma, as shown in Table 3, Nos. 5 to 12, incl. Therefore, it is clear that the failures at the time of the first test were the result of deficient luteal tissue and not of deficiency of LTH secretion. Corpora lutea present at the time of pituitary transplantation thus must have lost or have been in the process of losing their reactivity.

Series II-B-2 gave results that were in sharp contrast to this (Table 4).

TABLE 3. PITUITARY TRANSPLANTATION TO THE KIDNEY CAPSULE ON THE DAY OF PROESTRUS (SERIES II-B-1)

Animal No.	Days, operation to 1st trauma	Decidual response	Days, operation to 2nd trauma*	Decidual response
1	4	0	—	—
2	4	0	—	—
3	7	0	41	0
4	6	0	54	0
5	6	0	40	+ to ++
6	12	0	58	++++
7	21	0	37	++++
8	21	0	48	++ & +++
9	19	0	46	++
10	15	0	37	+++
11†	5	0	40	++ & +++
12†	13	0	29	++++
13	7	++ & +++	—	—
14	15	++ & +++	—	—
15	15	++ & +++	—	—

*Shortly before 2nd trauma Nos. 3 through 12 were subjected to FSH-LH injections to form new corpora lutea.

† Right ovary was removed 4 days after right uterus was traumatized.

These 9 rats, in which pituitary transplantation was carried out on the third day of diestrus during 5-day cycles, produced strong decidual responses to the initial uterine trauma. Therefore, their corpora lutea remained responsive to LTH, although they were of identical age to those in II-B-1 at the time of the transplantation operation.

OVARIES. The ovarian histology which one finds in rats after the pituitary has been transplanted to the kidney capsule during estrus is so uniform that it can serve as a standard with which ovaries in the present

TABLE 4. PITUITARY TRANSPLANTATION TO THE KIDNEY CAPSULE ON THE THIRD DAY OF DIESTRUS (SERIES II-B-2)

Animal No.	Days, operation to 1st trauma	Decidual response
1	6	+++
2	5	+++
3	4	++++
4	10	++++ & +++
5	6	+++
6	9	++ & +++
7	9	++++
8	5	++++
9	6	++ & +++

series may be compared. As described by Everett (9) there is always a set of large, healthy persistent corpora lutea, which represent those that were current at the time of the transplantation. They are identical with the corpora lutea of a normal pseudopregnant rat in size (ca. 1.5 mm. diam.), gross appearance and histological structure (Fig. 1-A) They are designated CL_I. Smaller ones which are thought to be the next older set are also present and designated CL_{II}. The interstitial tissue and follicular apparatus are uniformly atrophic.

This standard type of ovary was seen in all animals of Series I, the anterior eye-chamber series, at times when positive decidual responses were in evidence (Fig. 1-B). With the failure of such responses the corpora lutea were found to be in poor condition or were absent.

In Series II-A, transplantation to the kidney during estrus or diestrus day 2, the ovaries corresponded to the standard in every case (Figs. 1-C and 2-A). On the other hand, in Series II-B certain differences were seen. The most extreme were in Series II-B-1, proestrus transplantation. While results of the first uterine trauma were being assessed it was noted that in the 12 negative cases, although corpora lutea were present, they were relatively small (1.2 mm. or less). Two ovaries, one from each of two animals, were removed at that time for histological study. Their corpora lutea (Fig. 1-E) contained parenchymal cells that were generally reduced in size, and there was a conspicuous increase in numbers of stroma cells

along the sinusoids (Fig. 2-B). As noted earlier, these corpora lutea were not sufficiently active to sustain deciduoma reactions.

In Series II-B-2, transplantation on diestrus day 3, in correlation with the positive deciduoma tests, there were corpora lutea which more closely

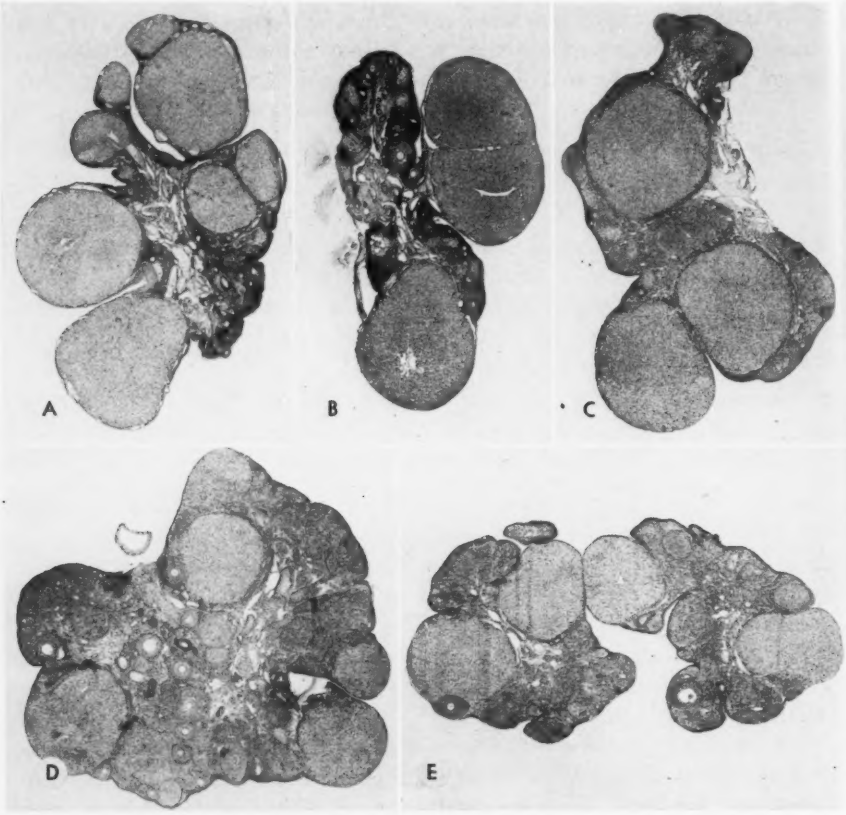


FIG. 1. A. Standard appearance of ovary from an animal with a pituitary graft in the kidney after transplantation during estrus, reproduced from Everett (9, Fig. 3-A). The presence of large and small sets of corpora lutea, the uniform atrophy of the interstitial tissue and follicular apparatus are the general features. (×15).

B. Ovary from animal with a pituitary graft in the eye. Transplantation during estrus. Note the similarity to Figure 1-A. (×15).

C. Ovary from animal with a pituitary graft in the kidney. Transplantation on diestrus day 2. Note the similarity to Figures 1-A and 1-B. (×15).

The smaller set of corpora lutea is lacking only from chance in the sections selected for B and C.

D. Ovary from a rat with a pituitary graft in the kidney. Transplantation on diestrus day 3. Note that the corpora lutea are smaller than in the preceding figures. (×15).

E. Ovary from a rat with a pituitary graft in the kidney. Transplantation during proestrus. Note the similarity to Figure 1-D. (×15).

resembled the standard in histological appearance (Fig. 2-C) than those just described. The parenchymal cells were more uniformly plump and there was no appreciable increase of stroma cells. In size, however, these corpora lutea were mostly smaller than CL_I of the standard, as one can readily note in Figure 1-D.

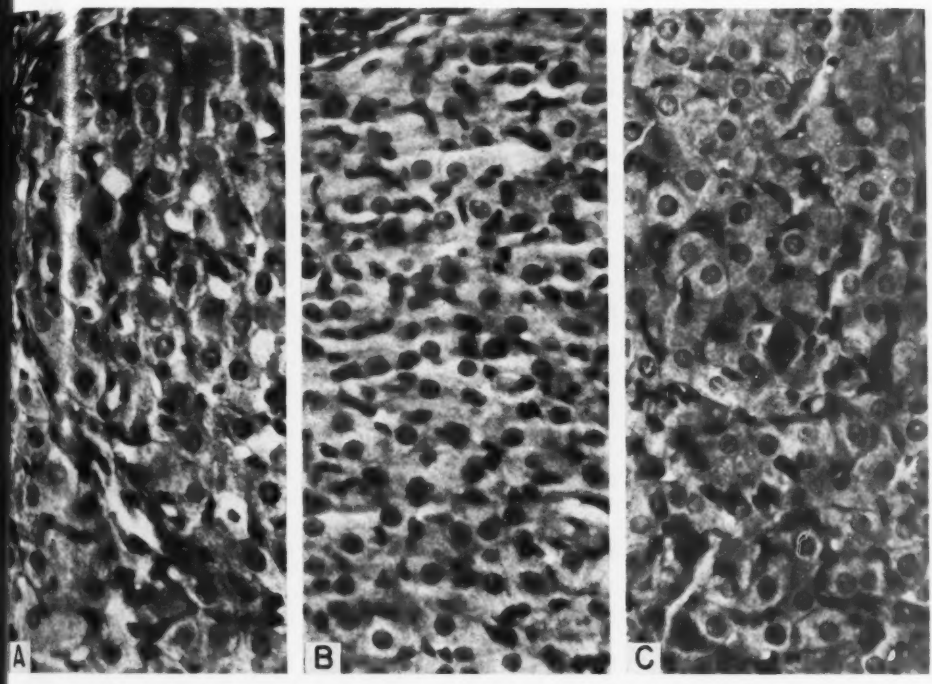


FIG. 2. A. Luteal cells from ovary shown in Figure 1-C. The healthy appearance and the large size of these cells are so similar to those of the standard that an additional figure of the latter was judged unnecessary. (×400).

B. Luteal cells from ovary shown in Figure 1-E. Note the reduced size of parenchymal cells and the great increase in numbers of stroma cells. (×400).

C. Luteal cells from ovary shown in Figure 1-D. Although the age of this corpus luteum was about the same at the time of transplantation as that in the preceding picture, the parenchymal cells here are larger, the stroma cells are not increased in number and the general appearance is very similar to that of Figure 2-A. (×400).

PITUITARY GRAFTS. In the case of transplantation to the kidney capsule the "take" of grafts was 100%, no failure having ever been encountered. The grafts were always large and very well vascularized with well opened sinusoids, regardless of the duration of the experiment. They were always composed of small, relatively undifferentiated cells. The eye transplants had a similar cellular composition but their general aspect was

not as uniform as that of kidney grafts. The transplants to the eye taken from animals that had functional corpora lutea throughout the experiment (Series I-A) were of moderate size and vascularization, but in neither quality were they equal to any of the kidney grafts. Eye grafts from animals that had functional corpora lutea at the beginning but not at the end of the experiment (Series I-A) were always small and very poorly vascularized. In animals with non-functional grafts (Series I-B), the eyes bearing the grafts were examined at autopsy with a dissecting microscope and, in no case, could any appreciable amount of glandular tissue be found.

DISCUSSION

This work demonstrates once more that in the rat the adenohypophysis when transplanted to a site distant from the diencephalon will lose its ability to secrete FSH and LH and at the same time will retain or increase its secretion of LTH. The site of transplantation does not seem to influence this outcome unduly in qualitative aspects. It is now apparent that grafts placed in the anterior chamber of the eye produce LTH in amounts sufficient to support corpus luteum function. This effect was not encountered in the work of earlier investigators simply because they employed hypophysectomized rats into which homotransplants of hypophyses were introduced at a later date. By that time the ovaries unquestionably lacked corpora lutea that could respond.

Although intraocular transplants of pars distalis are now seen to be capable of secreting LTH, it appears that the kidney offers more favorable conditions for a graft to "take" (sufficient space, vascularization, optimum temperature). This is true when kidney grafts are compared not only to eye grafts but also to grafts in the fascia of the neck (8) or in the connective tissue outside the kidney capsule. The kidney grafts are always larger and very well vascularized, retaining the typical sinusoids found in the gland *in situ*, well injected with erythrocytes. On the other hand, grafts in the eye are often very small, their vascularization is scanty and the number of erythrocytes seen in the sections, few. The anterior chamber of the rat eye at best offers a very restricted space, which may largely account for much of the difference. In addition, one should recall that the gland was manipulated to a greater extent during transplantation to the eye and this also may have contributed to the observed deficiency.

The secretory capacities of the grafts were not influenced by the time of the cycle when the pars distalis was transplanted, estrus, diestrus or proestrus. In fact, even the hypophyses of new-born rats have proven capable of secreting LTH when transplanted into the kidney capsule of the hypophysectomized mother (unpublished). Hence, it would appear that the state of the gland when transplanted does not determine the character of its later activity.

Transplantation of the pituitary under the conditions of the presen

experiments does not induce formation of new corpora lutea. This is clearly demonstrated by Series II-B-1 in which the operation took place very near the normally expected time of LH release (13). Although at that time the grafted gland must hold its maximum content of LH, absorption of the hormone from the transplant can be presumed to be too slow to be effective.

Information about the functional status of corpora lutea at different stages of the cycle is furnished by the results of Series II. As late as 50 to 60 hours after ovulation the corpora lutea retain the capacity to be brought to full secretory function. They have proven to be equally responsive whether the hypophysis is transplanted during estrus or the second day of diestrus and are similar in every feature to corpora lutea of normal pseudopregnancy. Only in very late diestrus does responsiveness of the corpora lutea begin to wane, as indicated by the fact that they cannot be stimulated to an increase in size comparable to that in the preceding groups. The responsiveness then becomes irretrievably lost during proestrus,[4] at which time one may say that luteolysis is well advanced. This statement is in accord with reports that patches of fatty necrosis appear in rat corpora lutea during proestrus (14, 15, 16, 17).

Corpus luteum age itself is certainly not responsible for luteolysis. In the first place, luteolysis is interrupted or prevented by the transplantation of the hypophysis (8, 9). Furthermore, in the two parts of Series II-B (transplantations during proestrus and diestrus day 3, respectively) the corpora lutea were of equivalent age at operation, yet they had regressed far more in the former instance. One must conclude, therefore, that luteolysis reflects certain hormonal changes specifically associated with proestrus. Whatever these changes may be, their first effect appears late in diestrus. No attempt was made in the present work to identify the substance(s) responsible for luteolysis. Others have suggested (18, 19) that the combination of FSH and LH may serve in this manner.

The length of time during which the endometrium retains its reactivity to trauma was unexpected (42 days in this experiment and as long as 74 days in unpublished cases). In fact, Everett (9) purposely avoided the deciduoma test in long-term experiments because it seemed unlikely that the uterus would respond several weeks after pituitary transplantation. During normal pseudopregnancy in the intact rat, the reactivity is lost after the seventh day (11, 12). The general belief has been that only when the uterus has recently been stimulated by estrogen can progesterone support a decidual reaction (20, 21). The atrophic state of the follicular apparatus, interstitial tissue and vaginal epithelium indicate, however,

[4] The three exceptional animals in the proestrus transplantation group which had functional corpora lutea are assumed to have been not in true proestrus, in spite of the vaginal smear record. This can be expected in a small proportion of rats in this colony at any given time, and true proestrus will be found on the following day.

that estrogen secretion is essentially absent in animals with pituitary grafts.

An additional finding made possible by the special characteristics of these experiments is that the existence of an earlier deciduoma, or several of them, will not preclude an equivalent response to a second trauma. Futhermore, the pituitary engrafted female rat is a potentially valuable preparation for the study of ageing of a deciduoma under relatively constant hormonal conditions. Material at hand is inadequate for this and allows one to say only that deciduomata do regress within a few weeks.

Acknowledgment

We are indebted to Mr. Carl Bishop of the Department of Pathology for the preparation of photomicrographs.

REFERENCES

1. Martins, T.: *Compt. rend. Soc. biol.* **123**: 699. 1936.
2. Richter, C. P. and J. F. Eckert: *Endocrinology* **21**: 481. 1937.
3. Westman, W. and D. Jacobsohn: *Acta Path. Microbiol. Scandinav.* **17**: 328. 1940.
4. Cheng, C. P., G. Sayers, L. S. Goodman and C. A. Swinyard: *Am. J. Physiol.* **159**: 426. 1949.
5. Fortier, C.: *Endocrinology* **49**: 782. 1951.
6. Fortier, C. and H. Selye: *Am. J. Physiol.* **159**: 433. 1949.
7. Desclin, L.: *Ann. d'Endocrinologie* **11**: 656. 1950.
8. Everett, J. W.: *Endocrinology* **54**: 685. 1954.
9. Everett, J. W.: *Endocrinology* **58**: 786. 1956.
10. Desclin, L.: *Ann. d'Endocrinologie* **17**: 586. 1956.
11. Long, J. A. and H. M. Evans: *Memoirs Univ. California* **6**: 1. 1922.
12. Allen, W. M.: *Anat. Rec.* **48**: 65. 1931.
13. Everett, J. W.: *Endocrinology* **59**: 580. 1956.
14. Boling, J. L.: *Anat. Rec.* **82**: 131. 1942.
15. Bassett, D. L.: *Am. J. Anat.* **73**: 251. 1943.
16. Everett, J. W.: *Am. J. Anat.* **77**: 293. 1945.
17. Deane, H. W.: *Am. J. Anat.* **91**: 363. 1952.
18. Greep, R. O.: *Endocrinology* **23**: 154. 1938.
19. Greep, R. O., H. B. van Dyke and B. F. Chow: *Endocrinology* **30**: 635. 1942.
20. Weichert, C. K.: *Proc. Soc. Exper. Biol. and Med.* **25**: 490. 1928.
21. Courrier, R.: *Vitamins and Hormones* **8**: 179. 1950.

NOTES AND COMMENTS

SYNERGISTIC ACTIONS OF MEPROBAMATE AND AN ANTICHOLINERGIC DRUG, PATHILON, ON REPRODUCTIVE CYCLES IN THE RAT

ABSTRACT

In 10 adult female albino rats the combined administration of meprobamate and Pathilon, an anticholinergic drug, each in a dosage not greatly effective by itself on the reproductive cycle in other similar groups, leads to an augmentation of their actions. Thus the blocking effectiveness with respect to ovulation was 75% (significant at the 5% level). Furthermore, in 8 of 10 animals the subsequent diestrus was prolonged; 7 of them were pseudopregnant.

In long-term treatments of adult female rats with meprobamate (Gitsch, unpublished) a prolongation of diestrus took place in a small percentage of animals. At the same time it was observed that certain effects of norepinephrine (1) and chlorpromazine (2, 3) on the reproductive cycle are increased by combination with meprobamate. The recent finding (4) that the anticholinergic drug Pathilon is able to block spontaneous ovulation in adult rats similarly to atropine (5) and Banthine (6) raised the question whether meprobamate might possibly increase the effectiveness of Pathilon on this and other aspects of the cycle.

MATERIALS AND METHODS

The animals used were 36 female adult albino rats, belonging to an inbred line of the Osborne-Mendel strain. They weighed 180–220 gm. and showed vaginal cornification at regular 4-day intervals. At least 3 such cycles in sequence immediately preceding the experimental period were required of each rat.

The two drugs and their special characteristics are as follows: Firstly, meprobamate (Miltown, Lederle) is an interneural blocking agent used for tranquilizing purposes. The solution for injection was prepared by dissolving 100 mg. powder[1] in one cc. physiological saline. Because of low solubility the saline was warmed (about 40–50° C) before adding powder. Secondly, 3-diethylamino-1-cyclohexyl-1-phenyl-1-ethiodide (Pathilon, Lederle) is an anticholinergic drug, more potent than Banthine and with a wider therapeutic margin of safety. For injection 25 mg. of the powder[1] was dissolved in 0.5 cc. physiological saline.

The solutions were injected subcutaneously in the lumbar region. The time of injection was consistently on the day of proestrus between 1:20 and 1:30 P.M., about half an hour before the beginning of the "critical period" (7). The experimental rats were divided into 3 groups of 10. In Group I each rat received 100 mg./kg. Miltown; in Group II each rat received 25 mg./kg. Pathilon; and in Group III each rat received both drugs in these respective dosages. A control Group C of 6 rats was injected with physiological saline in the same volume as Group III.

All animals were laparotomized on the following day (estrus) for evidence of ovulation—observation of ruptured follicles in ovaries *in situ* under the dissecting microscope and presence of ova in the excised tubes (4, 8). The abdominal walls were repaired and the animals were kept under observation for an extended period. Whenever there was a prolongation of diestrus, the uterus was traumatized on the fourth diestrus day to test for pseudopregnancy. On the ninth day laparatomy was again performed to look for deciduomata. In every second animal at this time one ovary was removed for histological study. The routine staining procedure was a modified Mallory tri-acid technique (9).

RESULTS AND DISCUSSION

In Group I (Miltown), in 8 of the 10 rats ovulation occurred at the normal time.

Received July 31, 1957.

[1] Kindly supplied by the Lederle Laboratories Division, Pearl River, New York.

In 6 of them the following 3 cycles were normal. In the other 2 rats there was subsequently a prolongation of diestrus (14 days); one of them was pseudopregnant. In one rat ovulation was blocked (i.e. postponed) one day and in a second there was a partial block (only 4 tubal ova). The following 3 cycles were normal.

In Group II (Pathilon), in 7 of 10 rats ovulation occurred at the normal time and the three following cycles were normal. In 2 rats ovulation was blocked (postponed) one day; in one of them there followed a pseudopregnancy. In a third rat a partial blockade (only 3 tubal ova) was observed, after which there was a simple prolongation of the subsequent diestrus (12 days). This group is identical with the first group in Series I described by Gitsch and Everett (4).

The respective capacities of the two drugs for blocking are thus very low at the dosages used, 15%[2] for Miltown and 25%[2] for Pathilon. The diestrus following ovulation was prolonged in both Groups I and II in 20% of the cases (Table 1).

TABLE 1

	Groups and treatments			
	C Saline	I Meprobamate	II Pathilon	III Meprobamate + Pathilon
Percentage* blockade of ovulation	0	15	25	75
Percentage prolongation of next diestrus†	0	20 (10)	20 (10)	80 (80)

* See Footnote 2.
† Corresponding percentages of pseudopregnancies in parentheses.

In Group III (Miltown & Pathilon) all 10 animals were affected in some way. Ovulation was completely blocked in 7 rats. In 4 of them the ovulation was postponed only one day; in the remaining 3 rats, 2 days. Among these 7 blocked rats, 4 subsequently responded with a pseudopregnancy, 1 with a simple prolongation of diestrus (14 days), and 2 with normal cycles only. One other animal was partially blocked, but this was followed by successive pseudopregnancies. In the 2 remaining animals ovulation was not influenced, but both promptly became pseudopregnant. In conclusion, ovulation was blocked in 75% of this group, a figure which is significant at the 5% level (10) in terms of expected frequency of spontaneous delay of ovulation (7). Furthermore, 8 of the 10 rats had a prolongation of following diestrus, in which case 7 rats proved to be pseudopregnant (Table 1). Histologically, in the pseudopregnant animals the ovaries disclosed nothing different from what one could expect to find in normal pseudopregnancy. The corpora lutea were large and well developed.

In the control Group C injected with physiological saline, normal ovulation was observed and the subsequent cycles were normal.

Thus, in two respects the two drugs augmented each other in their effects on the reproductive cycle. The frequencies of blocked ovulation and of effects on the subsequent diestrus were increased in comparison with the corresponding effects of either drug separately. The results are comparable with those produced by larger amounts of Pathilon alone (4). The ability to block ovulation means an LH depression. Higher dosages of Pathilon by itself have depressive effects on FSH release (4); it was suggested that such depression might consequently lead to an increase of luteotropin. In the present

[2] In calculating such percentage, a case of partial blockade is arbitrarily given a value of 5% and one of total blockade receives a value 10%, one animal equalling 10% of the total.

experiments, the high proportion of pseudopregnancies in Group III suggests a similar action.

EDUARD GITSCH[3,4]

Department of Anatomy,
Duke University, School of Medicine,
Durham, North Carolina

Acknowledgment

The author wishes to express his great indebtedness to Dr. John W. Everett for his counsel while this work was in progress.

REFERENCES

1. SWINGLE, W. W., P. SEAY, J. PERLMUTT, E. J. COLLINS, G. BARLOW, JR. AND E. J. FEDOR: *Am. J. of Physiology* **167:** 586. 1951.
2. KRAIS, W., H. LEHR AND W. RIESS: *Arch. Gynäk.* **185:** 40. 1954.
3. BARRACLOUGH, C. A.: *Anat. Rec.* **127:** 262. 1957.
4. GITSCH, E. AND J. W. EVERETT: *Endocrinology* (in press).
5. EVERETT, J. W. AND C. H. SAWYER: *Endocrinology* **52:** 83. 1953.
6. SAWYER, C. H., J. E. MARKEE AND J. W. EVERETT: *Am. J. Physiol.* **166:** 223. 1951.
7. EVERETT, J. W. AND C. H. SAWYER: *Endocrinology* **47:** 198. 1950.
8. EVERETT, J. W.: *Endocrinology* **41:** 364. 1947.
9. EVERETT, J. W.: *Endocrinology* **32:** 285. 1943.
10. MAINLAND, D. AND J. M. MURRAY: *Science* **116:** 591. 1952.

[3] Lederle fellow in Endocrinology.

[4] Present address: II. Universitäts-Frauenklinik, Wien IX., Spitalgasse 23, Österreich (Austria).

RADIOIODINE CONCENTRATION AND THYROID WEIGHT IN NORMAL, OBESE AND DWARF STRAINS OF MICE[1]

ABSTRACT

Thyroid activity was measured in Rockland Swiss, obese-hyperglycemic and a strain of mice with a recessive gene for dwarfism. The thyroid glands of the Swiss mice were significantly heavier than those of the other strains and the phenotypic dwarfs had the smallest glands. The greatest I^{131} uptake occurred in the female Swiss mice and there was relatively little iodine concentration in the dwarf thyroids. However, the phenotypically normal parent stock of the obese or dwarf strains did not differ significantly from the Swiss mice. The phenotypically obese mice tended to concentrate less I^{131} during a five hour period but the differences were not statistically significant.

Much research has been done on strain differences in thyroid function in the domestic fowl (1, 2, 3, 4, 5) and in mice (6, 7, 8, 9, 10). The relationships of hypothyroidism to lipid metabolism in rats and mice has been investigated (11, 12) and the effects of a mild experimentally produced hyperthyroidism on the growth rate of mice, rats, the guinea pig and rabbit has also been studied (9, 12). This paper reports the results of a

Received October 31, 1957.

[1] Journal Paper No. 1176, Purdue University Agricultural Experiment Station.

study of thyroid weight and I^{131} uptake in three strains of mice with widely different genotypes.

MATERIALS AND METHODS

One of the strains was a stock of Rockland Swiss mice maintained at Purdue for more than 10 years for estrogen assays. The other two strains were originally obtained from the Jackson Memorial Laboratory and had been maintained at Purdue for about three years.

One strain is described as obese-hyperglycemic. The parent stock were heterozygous for obesity and of the genetic composition *Ob ob*. The double recessive mice of this strain are extremely obese and have the genetic makeup *ob ob*. The third strain carries a recessive gene for dwarfing. The parent stock were heterozygous, *Dw, dw*, and compared rather favorably in size with the Swiss and normal mice of the obese strain. The double recessive dwarfs were extremely small, of low vigor, and difficult to raise. As a result, few were available for study.

Iodine concentration in the thyroid was determined five hours following the intraperitoneal injection of 1.0 microcurie of carrier-free I^{131} as NaI^{131} in aqueous solution. This level of activity and time interval were chosen following preliminary studies with levels from 0.25 to 3.0 microcuries and time intervals of two to 24 hours. At autopsy the radioactivity of the individual glands was measured with the R1DL Model 230 Scintillation Spectrometer. The differential method of counting I^{131} at the 0.364 Mev. gama-emission peak was used. The I^{131} values were expressed as per cent of uptake of the administered dose at a five-hour interval following administration.

It is recognized that many factors influence thyroid activity and that per cent iodine uptake at a particular time has definite limitations. In order to minimize some of the sources of variation the breeding colony was maintained in an air-conditioned room at approximately 24° C. The animals received a ration containing a known amount of idone, 0.235 μg. of iodine per gram. The test animals were kept at a temperature of approximately 25° C prior to and during the uptake period and were between 8 and 10 weeks of age at autopsy. Plasma and urinary radioiodine concentrations were not measured. However, all strains of mice and both sexes were compared on the same basis.

The mice were confined in individual cages without food or water during the uptake period. At exactly five hours they were weighed, chloroformed, and the thyroid glands removed and weighed. In the case of the phenotypic dwarf mice the glands were extremely small and weighed less than one milligram. Because of the difficulty in obtaining cleanly dissected glands of this size they were not routinely weighed. This was not an important factor in the accurate determination of I^{131} activity. In other studies counts of cleanly dissected thyroids were compared with those in which the glands were attached to a small section of the trachea. There was a difference of less than 1% in total radioactivity attributable to the presence of the trachea.

The data on thyroid weights and body weights were first analyzed statistically by the method of analysis of variance; the Duncan-Bonner Test was then applied to detect significant differences between mean values. The data on per cent uptake of I^{131} by the thyroid glands were analyzed by the Student-Fisher t-Test.

RESULTS

The data are summarized in Table 1. There were highly significant differences in body weight and thyroid weight between the three strains of mice ($P < 0.01$).

The thyroid weights of the Rockland Swiss mice were significantly heavier than those of the obese and dwarf strains ($P < 0.01$). The thyroids of the phenotypically normal dwarf-strain males were significantly heavier than those of the phenotypically normal male and female mice of the obese strain ($P < 0.01$). Also, the thyroids of phenotypically obese mice were heavier than those of the phenotypically normal mice of the obese strain, but not significantly so. Although the thyroid weights of the females in general tended to be slightly lighter than those of the males in each strain, the weight differences were significant only between the phenotypically normal males and females of the dwarf strain ($P < 0.01$).

As expected the phenotypically normal females of each strain tended to be significantly smaller in body size than the phenotypically normal males ($P < 0.01$). Differences in the body weights of phenotypically obese mice and phenotypically dwarf mice were highly different from the body weights of all the other mice, as expected ($P < 0.01$). There was no significant body weight difference between the phenotypically obese males and females of the obese strain.

Phenotypically normal mice of the obese strain were significantly smaller in body size than Rockland Swiss mice ($P < 0.01$) except in the comparison of Rockland Swiss females with phenotypically normal males of the obese strain. There were, however, marked similarities in body size between the phenotypically normal males of the obese strain and the phenotypically normal males of the dwarf strain.

When thyroid weight was expressed as a per cent of body weight, as shown in Table 1, there was no difference between the Rockland Swiss and the phenotypically normal mice of the dwarf strain. The obese mice, on a percentage of body weight basis, had somewhat smaller thyroid glands than the other groups with the exception of the dwarfs.

TABLE 1. COMPARISON OF MEAN VALUES FOR BODY WEIGHTS, THYROID WEIGHTS AND I^{131} UPTAKE BY THYROIDS IN THREE STRAINS OF MICE

Strain	Genotype	Sex	Total No. mice	Body wt. (gm.)	Thyroid wt. (mg.)	Thyroid wt. as % body wt.	I^{131} uptake in 5 hours (%)
Rockland Swiss	All Same	Male	33	28.6 ± 2.7*	4.58 ± 0.87	0.016 ± 0.003	7.8 ± 2.9
	All Same	Female	35	23.4 ± 2.3	4.31 ± 0.73	0.018 ± 0.003	9.5 ± 2.8
Obese (*ob*)	Ob Ob or Ob ob	Male	17	20.9 ± 2.1	2.78 ± 0.42	0.013 ± 0.002	8.7 ± 4.0
	ob ob	Male	12	32.9 ± 4.8	3.25 ± 0.51	0.010 ± 0.003	6.5 ± 3.8
	Ob Ob or Ob ob	Female	19	18.4 ± 1.9	2.60 ± 0.42	0.014 ± 0.002	6.1 ± 2.4
	ob ob	Female	15	34.2 ± 6.4	3.11 ± 0.49	0.009 ± 0.002	4.8 ± 1.7
Dwarf (*dw*)	Dw Dw or Dw dw	Male	30	22.1 ± 3.1	3.55 ± 0.57	0.016 ± 0.003	8.0 ± 4.6
	Dw Dw or Dw dw	Female	23	17.6 ± 1.9	2.89 ± 0.71	0.016 ± 0.004	5.5 ± 3.1
	dw dw	Both	8	6.0 ± 0.9	—	—	0.4 ± 0.2

* The values given are the means and the standard deviations.

Statistical analysis of the I^{131} uptake data showed that the phenotypic dwarf mice had by far the lowest rate of uptake ($P < 0.01$). When the data were grouped by sexes, excluding the phenotypically dwarf mice, there was no significant difference between males and females. I^{131} uptake was significantly greater ($P < 0.01$) in the Swiss females than in the females of the other two strains. The uptake of I^{131} was not significantly different between the phenotypically obese mice and the phenotypically normal mice of the obese strain.

DISCUSSION

The above data contribute additional evidence of differences in thyroid function between various strains of mice and in this sense substantiate the earlier findings of Koger *et al.* (7), Hurst *et al.* (9) and Stob *et al.* (10). The results on I^{131} uptake in the phenotypically normal and obese mice of the obese-hyperglycemic strain are in agreement with those of Bleisch *et al.* (13) and Goldberg *et al.* (14) that the obesity is not associated with any marked difference in thyroid function.

The observation in the present study that there are significant differences in body weight and thyroid weight between the three strains without more differences in I^{131} uptake cannot be readily explained. It is clear that the Swiss females had significantly heavier thyroids and significantly greater I^{131} uptake than either the phenotypically normal or obese females of the obese strain and the phenotypically normal females of the

dwarf strain; but it seems equally clear that there is no consistent difference in I^{131} uptake between the males of the Swiss, obese or dwarf strains (with the exception of the phenotypic dwarfs). Noteworthy also is the similarity in growth rate and body size of the phenotypically normal males and females of the obese and dwarf strains, in contrast to the extreme obesity or dwarfism evidenced by the homozygous dwarf or obese mice.

A. A. Wykes,[2] J. E. Christian and F. N. Andrews

School of Pharmacy
Department of Animal Husbandry
Purdue University
Lafayette, Indiana

REFERENCES

1. Mixner, J. P., E. P. Reineke and C. W. Turner: *Endocrinology* **35**: 168. 1944.
2. Schultze, A. B. and C. W. Turner: *University of Missouri A. E. S. Research Bulletin 392*. 1945.
3. Mixner, J. P. and C. W. Upp: *Poultry Science* **26**: 389. 1947.
4. Glazener, E. W., C. S. Shaffner and M. A. Jull: *Poultry Science* **28**: 834. 1949.
5. Andrews, F. N., S. R. Miles and O. S. Davis: *Endocrinology* **52**: 712. 1953.
6. Boettiger, E. G.: *Endocrinology* **28**: 785. 1941.
7. Koger, M., V. Hurst and C. W. Turner: *Endocrinology* **31**: 237. 1942.
8. Koger, M. and C. W. Turner: *University of Missouri A.E.S. Research Bulletin Bulletin 377*. 1943.
9. Hurst, V. and C. W. Turner: *University of Missouri A.E.S. Research Bulletin 417*. 1948.
10. Stob, M. and F. N. Andrews: *Endocrinology* **51**: 165. 1952.
11. Scow, R. O.: *Endocrinology* **49**: 522. 1951.
12. Boell, E. J.: *Dynamics of the Growth Process*, Princeton University Press, Princeton, New Jersey, 203–206. 1954.
13. Bleisch, V. R., J. Mayer and M. M. Dickie: *Am. J. Path.* **28**: 369. 1952.
14. Goldberg, R. C. and J. Mayer: *Proc. Soc. Exper. Biol. & Med.* **81**: 323. 1952.

[2] Present address, Abbott Laboratories, North Chicago, Illinois.

A NEW APPARATUS FOR DETERMINING OXYGEN CONSUMPTION IN RATS[1,2]

ABSTRACT

An apparatus is described which can be easily constructed for student or research use and which gives excellent results in measuring the oxygen consumption of rats. Results obtained with the apparatus are similar to those reported elsewhere.

Holtkamp *et al.* (1) have described an apparatus for measuring the oxygen consumption of groups of rats in a single experiment. The basic idea is sound but the apparatus

Received November 21, 1957.

[1] Supported by the National Institute for Arthritic and Metabolic Diseases, Grant A-897.

[2] Thanks are due Dr. John Haldi and Mr. Winfrey Wynn for much of the testing of this apparatus.

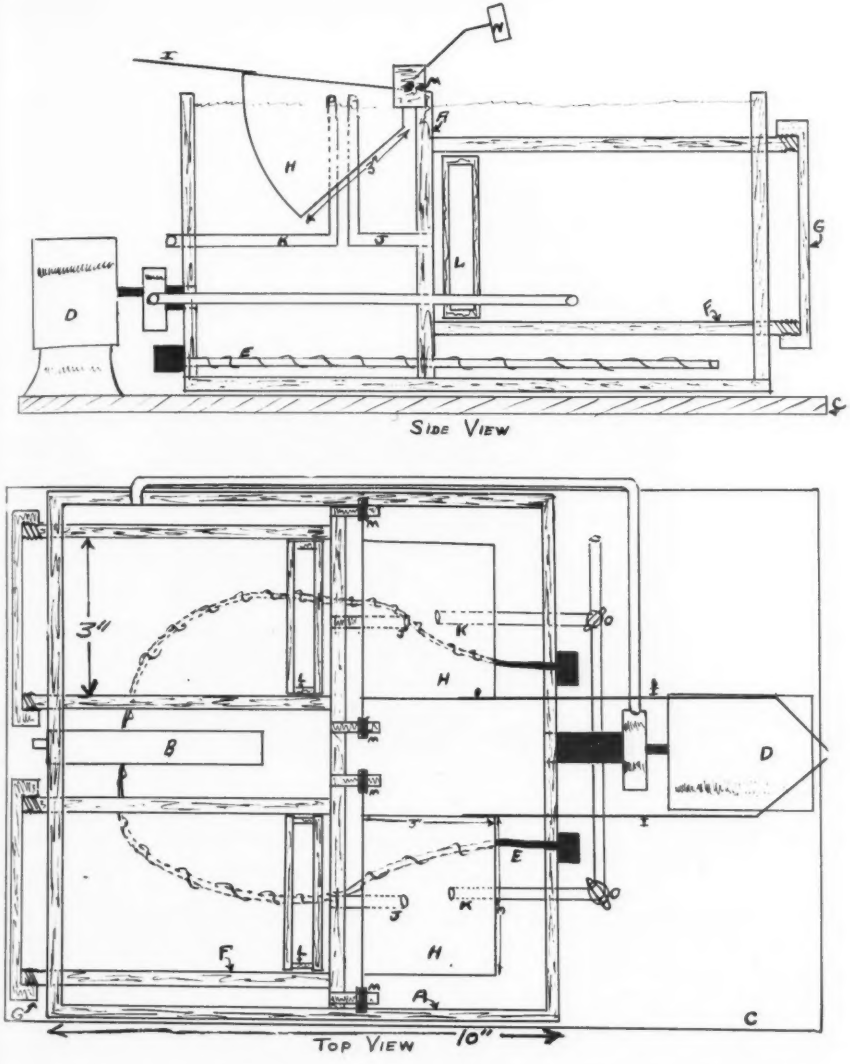


FIG. 1. A diagram of an apparatus for measuring oxygen consumption in small animals. Most of the dimensions are not critical and are not indicated.

is not satisfactory because it requires constant attendance, does not give permanent records, and is cumbersome to operate in removing and replacing animals.

We have redesigned this apparatus in such a manner that all of these difficulties are avoided.

METHODS

The apparatus is presented in Figure 1. It was constructed of readily available materials and can be made by any competent shop man. The instrument is constructed of plastic for easy assembly and for complete vision. No dimension is critical and the size can be varied to

suit the animal to be tested. The method of construction is as follows: A box was constructed from ¼ inch plastic which is 10×10 inches square with sides 5½ inches high and partition (A) of ¼ inch plastic is constructed across the box at approximately the half-way point with holes for water circulation, cut-outs for heater coils, and holes for connections to the spirometers. This assembly constituted the water bath.

The bath was mounted on a board (C) slightly longer than the instrument and a circulating pump (D) (centrifugal pump supplied by Herbach and Rademan, Phila.) was fastened to the board. Connections were made as illustrated from the pump to the bath so that water

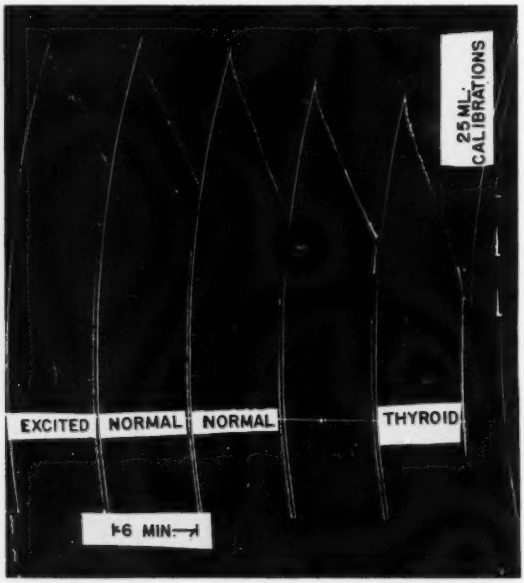


FIG. 2. Results obtained with the apparatus on the oxygen consumption of rats. All consumptions were measured over a six minute period. The effect of movement, normal oxygen consumption, and the oxygen consumption of a hyperthyroid rat (given 100 mcg. TRIT) is clearly shown.

could be circulated continuously. A heatercoil (E) (Fisher, 100 watt) was coiled in the bottom of the box and the ends taken out through the wall to a 110-volt A.C. connection. A Fenwall thermoregulator (B) was installed in the line for temperature control. A constant water level can easily be installed if desired.

The animal containers were easily made from plastic tubing (F) in the desired diameter which may be varied according to the size of animal to be tested. For the rat, three inch tubing was satisfactory. The tubing was threaded on one end and a cap (G) was cut from sheet plastic (½ inch) and threaded to fit the tube. A hole was now cut through the wall of the water bath to fit the outside diameter of the tubing, the tube passed through, cemented to the partition, and the joints were made air-tight by a liberal application of plastic cement.

The spirometers were made of thin shim brass (H) cut to the desired dimensions, suspended on jeweled bearings (M), and counter-weighted with small copper weights (N). The critical dimensions are on the drawing. A pointer (I) was soldered to the swinging edge and arranged to write on a kymograph drum. Connections (J, K) were supplied from the animal container to the spirometer and from the spirometer to the oxygen supply through small stopcocks (O).

A small cylinder (L) of plastic was cut to fit the inside of the animal holder, drilled with many small holes, and filled with soda lime to absorb carbon dioxide.

Although the apparatus was built with two holders for experimental and control animals, it could be built with many containers. The present plan permits the introduction and removal of the animal without disturbance of the bath or the spirometer. It also permits extremely short connections between holder and spirometer to lessen any dead space effect.

The operation of the apparatus is extremely simple. After the animal has been placed in the container and the end sealed, oxygen is introduced in carefully measured amounts with a ten ml. oiled syringe, using a three-way stopcock. The movement of the pointer is registered on the kymograph drum and can be measured to determine the exact relationship between volume and movement of the spirometer. Oxygen is now introduced, the system is closed and the downward movement of the pointer records the oxygen used by the animal. A control and an experimental animal may be run simultaneously. Simple calculations determine the oxygen consumption and this may be corrected for barometric pressure and for water vapor as desired.

RESULTS

Figure 2 illustrates results which have been obtained in this apparatus. It will be observed that there is a regular fall of the pointer permitting easy calculation of oxygen consumption. The administration of a drug which alters oxygen consumption is seen to produce dramatic changes in the record indicating the sensitivity of the method. The results we have obtained for oxygen consumption agree very well with those of Holtkamp *et al.* Five adult rats had an oxygen consumption of 1.46 ml./hr./gm. B.W. ±S.D. 0.10 and 5 thyroidectomized animals had a consumption of 1.10 ±S.D. 0.08 ml./hr./gm. B.W.

HAROLD BREDING AND J. H. U. BROWN

Department of Physiology
Emory University
Georgia

REFERENCE

1. HOLTKAMP, D. E., S. OCHS, C. C. PFEIFFER AND A. E. HEMING: *Endocrinology* **56**: 93. 1955.

NONSPECIFICITY OF THE MECHANISM THAT ELICITS MYOCARDIAL NECROSES IN HUMORALLY CONDITIONED RATS

ABSTRACT

Following pretreatment with 2-methyl-9(α)-chlorocortisol and NaH_2PO_4, massive, multiple myocardial necroses can be precipitated by a variety of stressor agents; hence, this eliciting mechanism is considered to be largely nonspecific.

A brief period of neuromuscular effort regularly precipitates multiple myocardial necroses in rats pretreated with small doses of 2-methyl-9(α)-chlorocortisol (Me-Cl-COL) and an excess of NaH_2PO_4 (1). We wished to determine whether such infarct-like foci can also be elicited by other stressors in suitably pretreated rats.

One hundred and forty female Sprague-Dawley rats, with a mean initial body weight of 96 gm. (range: 90–108 gm.), were subdivided into 14 equal groups (Table 1).

The rats of all groups were conditioned by treatment during five days with *Me-Cl-COL* (microcrystals of the acetate at the daily dose of 50 μg. in 0.2 ml. of water, subcutaneously) and *NaH_2PO_4* (15% aqueous solution, 2 ml., twice daily, by stomach tube). *Group I* acted as otherwise untreated controls. In all other groups additional treatments were administered only on the fourth day. The rats of *Group II* were forcefully restrained in the prone position during seven hours, by strapping their extremities to a board with adhesive tape. *Group III* was anesthetized by the intraperitoneal administration of 3 mg. of pentobarbital in 0.3 ml. of water, followed by a second injection of 1 mg. in 0.1 ml., five hours later when the animals began to awaken. In *Group IV*, both vagi were

Received November 25, 1957.

transected, near the bifurcation of the carotid arteries. This, like all other surgical operations, was performed under ether anesthesia. The rats of *Group V* were immersed in icy water during 3 min. and those of *Group VI* in water at +48° C, during 5 min., at 10:00 A.M., 1:00 P.M. and 5:00 P.M. In *Group VII*, the spinal cord was transected, with a thermocautery, just caudad from the seventh cervical vertebra. In *Group VIII*, the femur, tibia and humerus were fractured on both sides. In *Group IX*, the stomach, ilium and caecum were each crushed three times with a thin hemostat. In *Group X*, quadriplegia was produced by bilateral extirpation of the brachial plexus and transec-

TABLE 1. INDUCTION OF CARDIOPATHY BY VARIOUS STRESSOR AGENTS

Group	Treatment*	Cardiopathy		Mortality (0/0)
		Incidence (0/0)	Severity (Scale: 1–3)	
I	None	20	0.5±20	0
II	Restraint	100	**2.6**±22	70
III	Pentobarbital	30	0.8±0.41	0
IV	Vagotomy	60	**1.8**±0.45	70
V	Cold baths	70	**1.8**±0.41	60
VI	Hot baths	90	**2.1**±0.32	70
VII	Spinal cord transection	40	0.4±17	0
VIII	Bone fractures	70	**1.9**±36	10
IX	Crushing of intestines	100	**2.5**±0.20	10
X	Quadriplegia	100	**3.0**±0	30
XI	Adrenaline	60	**1.7**±0.40	10
XII	Noradrenaline	60	1.4±0.40	10
XIII	"Pitressin"	40	1.1±0.40	10
XIV	Nephrectomy	40	0.7±0.20	100

* Prior to the treatments indicated in this column, the rats of all groups were conditioned by Me—Cl—COL—Ac and NaH_2PO_4 as indicated in the text.

tion of the femoral obturator and sciatic nerves. *Group XI* received adrenaline hydrochloride, and *Group XII*, noradrenaline hydrochloride. Both groups received two subcutaneous injections at the dose of 200 μg. in 0.2 ml. of oil. *Group XIII* was given 10 pressor units of a posterior pituitary extract ("Pitressin," Parke, Davis), in 1 ml. of water, subcutaneously, twice. *Group XIV* was bilaterally nephrectomized.

The morphologic characteristics of the multiple necroses are illustrated in Figure 1.

Table 1 lists the incidence (judged by inspection of the whole heart with a loupe and histologic study of one section through each heart) and the mean severity of the cardiac lesions (expressed in an arbitrary scale of 1 to 3, on the basis of appraisal both by loupe inspection and histologic study) with its standard error. The readings printed in heavy numerals represent presumably significant ($P < 0.05$) aggravations of the cardiopathy, beyond the pretreatment level.

It is evident that several, entirely unrelated, agents proved effective in eliciting cardiac necroses. All nephrectomized rats died within 12 hours after the operation (presumably because of their inability to eliminate the orally administered phosphate); consequently, the relative mildness of the cardiopathy in this group is difficult to interpret. However, spinal shock, profound and prolonged pentobarbital anesthesia and sublethal doses of the pressor posterior lobe extract also failed to aggravate the cardiac lesions. Apparently, in the suitably conditioned animal, the precipitating mechanism can be activated by a great variety of stressors; yet, this activation is less nonspecific than some of the other manifestations of the general adaptation syndrome (e.g., the discharge of ACTH, or the involution of the thymicolymphatic organs), perhaps because

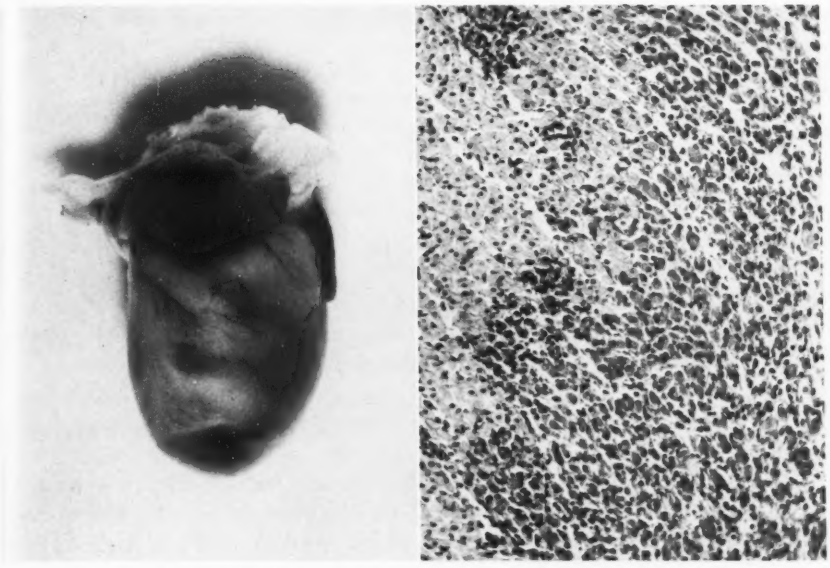


FIG. 1. *Left:* Macroscopic aspect of the heart of a rat in which extensive infarct-like necroses (pale areas) have been precipitated by neuromuscular effort after sensitization with Me-CL-COL and NaH_2PO_4. *Right:* Histologic aspect of the borderline between the healthy (upper left field) and the necrotic cardiac muscle of the same rat.

the nonspecific cardiotoxic action of some stressors can be counteracted by their specific effects upon the heart.

HANS SELYE, SERGE RENAUD AND MIKLOS NADASDI

Institut de Médecine et de Chirurgie expérimentales,
Université de Montréal, Montreal, Canada

Acknowledgments

These investigations were performed with the aid of a Consolidated Grant from the National Research Council of Canada and of a grant from the Muscular Dystrophy Association of Canada.

The authors are grateful to the Upjohn Company for generously supplying 2-methyl-9(α)-chlorocortisol.

REFERENCE

1. SELYE, H. AND RENAUD, S.: Humoral conditioning for the production of acute massive myocardial necroses by neuromuscular exertion, *Proc. Soc. Exper. Biol. & Med.* **96:** 512. 1957.

GORDON RESEARCH CONFERENCES

The Gordon Research Conferences for 1958 will be held from 9 June to 29 August at Colby Junior College, New London, New Hampshire; New Hampton School, New Hampton, New Hampshire and Kimball Union Academy, Meriden, New Hampshire.

Individuals interested in attending a Conference are requested to send their applications to the Director. Each applicant must state the institution or company with which he is connected and the type of work in which he is most interested. Attendance at each Conference is limited to 100.

The complete program of the Conferences will be published in *Science* for February 28th.

Requests for attendance at the Conferences, or for any additional information, should be addressed to W. George Parks, Director, Department of Chemistry, University of Rhode Island, Kingston, Rhode Island. From June 9 to August 29, 1958 mail should be addressed to Colby Junior College, New London, New Hampshire.

CONFERENCE ON THE CHEMISTRY, STRUCTURE AND PHYSIOLOGY OF BONES AND TEETH

To be held at Kimball Union Academy in Meriden, New Hampshire, July 14–18, 1958

D. DZIEWIATKOWSKI, Chairman; WALLACE D. ARMSTRONG, Vice Chairman

14 July

Elaboration of the Inorganic Structures in Bones and Teeth

REIDAR F. SOGNNAES, Chairman

J. T. ALBRIGHT and R. F. SOGNNAES. Fine Structure of the Inorganic Elements in Adult Bones and Teeth

S. F. JACKSON. Structural Relationship Between Inorganic and Organic Elements in Embryonic Bone

BAIRD HASTINGS, Chairman

HONOR B. FELL. The Study of Skeletal Physiology by the Organ Cluture Method

15 July

Physics of the Solid State

JOHN H. MARSHALL, Chairman

N. H. NACHTRIEB. Dynamics of the Solid State

WM. F. NEUMAN. The Bone Crystal

A. S. POSNER. Discussion

KARL MEYER, Chairman

HARRY BOSTROM. Aspects of the Metabolism of Mucopolysaccharides

16 July

Enzymes of Bone

ALEXANDER B. GUTMAN, Chairman
P. ROBBINS and F. D'ABRAMO. Active Sulfate and the Synthesis of Chondroitin Sulfate
H. F. DELUCA. Vitamin D and Citrate Metabolism
FRANKLIN C. MCLEAN, Chairman
NICOLAY EEG-LARSEN. Glycolysis in Epiphyseal Cartilage

17 July
Chemistry and Physiology of the Parathyroid Hormone
JOHN E. HOWARD, Chairman
H. RASMUSSEN. Studies on the Isolation of the Parathyroid Hormone
C. RICH, M. HORWITH, D. THOMPSON and H. RASMUSSEN. Physiological Effects of Purified Parathyroid Hormone in Humans
E. C. REIFENSTEIN, JR., Chairman
E. KODICEK. Metabolism of Vitamin D.

18 July
Current Research Trends
WALLACE D. ARMSTRONG, Chairman

SECOND WORLD CONGRESS, INTERNATIONAL FEDERATION OF GYNAECOLOGY AND OBSTETRICS

The Second World Congress of the International Federation of Gynaecology and Obstetrics, which will be held in the newly-built Queen Elizabeth Hotel in Montreal, Canada, from June 22 to 28, 1958, will have on its program eight main lectures given by eminent scientists whose studies and research are related to the field of gynaecology and obstetrics. The guest speakers and the title of their lectures are as follows:

PROFESSOR MURRAY L. BARR, Department of Microscopic Anatomy, University of Western Ontario, London, Canada: *Tests of Chromosomal Sex and Their Application to Clinical Problems.*

PROFESSOR HERMANN BAUTZMANN, Anatomisches Institut, Hamburg, Germany. *Comparative Studies on the Histology and Function of Animal and Human Amnion.*

PROFESSOR ROBERTO CALDEYRO-BARCIA, Chief of the Section of Obstetrical Physiology, Faculty of Medicine, Montevideo, Uruguay: *Contractility of the Human Gravid Uterus and Its Application to the Obstetrical Clinic.*

PROFESSOR G. W. HARRIS, Head of the Department of Neuroendocrinology, Maudsley Hospital, London, England: *Relationship of the Central Nervous System to Pituitary and Reproductive Activity.*

PROFESSOR CHARLES OBERLING, Institut de Recherches sur le Cancer Gustave Roussy, Villejuif (Seine), France: *The Cytology of the Cancerous Cell.*

PROFESSOR BRADLEY M. PATTEN, Chairman, Department of Anatomy, University of Michigan Medical School, Ann Arbor, Michigan: *The Establishing of Fetal-Maternal Vascular Relations.*

PROFESSOR HANS SELYE, Director of the Institute on Experimental Medicine and Surgery, University of Montreal, Montreal, Canada: *Stress in Gynaecology.*

A representative of the U.R.S.S. (on a subject of his choice)

All correspondence should be directed to the Montreal Committee, Second World Congress, International Federation of Gynaecology and Obstetrics, 1414 Drummond Street, Suite 220, Montreal 25, Canada.

REPRINTS WANTED ON ENDOCRINE-CANCER RELATIONSHIPS

The Cancer Chemotherapy National Service Center is seeking reprints for a reference collection covering the broadest range of endocrine-cancer relationships, as well as basic endocrinology. In particular it needs clinical data on cancer patients treated with hormones, data on the biological activities of hormones, analogs and antagonistic substances, and data on the effects of hormones on animal tumors. Assay data on steroids would be especially welcome. Those wishing to assist, please send reprints to: Dr. D. Jane Taylor, Endocrinology Section, Cancer Chemotherapy National Service Center, National Institutes of Health, Bethesda 14, Maryland.